Structural and functional boundaries of human polyclonal antibody responses against tetanus toxoid

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Structural and functional boundaries of human polyclonal antibody responses against tetanus toxoid

by

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PhD Thesis

Resumé (dansk)

Immunforsvaret arbejder konstant på at beskytte kroppen imod invaderende stoffer og sygdomsfremkaldende organismer som bakterier og virus (antigener). Antistoffer er vigtige molekyler, som immunforsvaret bruger til at genkende disse fremmedstoffer. Gennem den specifikke binding til antigener genkendes disse af effektorceller, som derefter kan eliminere det indtrængende antigen. Antistofmolekylets ene ende består af en konstant del, som ikke varierer meget imellem forskellige antistoffer, og som bruges til at kommunikere med kroppens immunceller. Den anden ende af antistoffet er meget forskellig imellem antistoffer, og det er denne del, som binder til de fremmede stoffer. Herved rammer dette sofistikerede system nærmest alt fremmed, der kommer ind i kroppen, ved at danne et repertoire af forskellige antistoffer. Når kroppen møder det samme antigen flere gange, modnes responset og antistoffernes bindingsstyrke til antigenet øges, hvilket resulterer i et mere effektivt respons..

Symplex-teknologien er den eneste metode, der kan isolere humane antistofrepertoirer. I løbet af mit ph.d.-arbejde har vi brugt denne teknologi til at isolere tre repertoirer imod stivkrampe fra hver af to frivillige donorer med mellem 1,5 til 4,5 års mellemrum. Desuden har vi isoleret et antistofrepertoire fra en donor, som for første gang modtog en stivkrampevaccination for at kunne sammenligne tidlige og sene repertoirer. Disse syv repertoirer er de mest omfattende specifikke antistofrepertoirer, der er rapporteret til dato, og det er det eneste studie, der følger udviklingen i antistofrepertoirer efter flere vaccinationer af de samme mennesker. Baseret på dette store antal antistoffer har vi rapporteret det første estimat af størrelsen på specifikke antistofrepertoirer. Vi har også – som de første – rapporteret, at antistofrepertoirer når et loft i bindingsstyrke efter flere vaccinationer. Desuden har vi fundet, at det antistofrespons, vi danner første gang, vi møder et bestemt antigen, ligner senere responser mere end hidtil antaget. Dog opnås det omtalte loft i bindingsstyrke først efter flere vaccinationer.

Forståelsen, der er opnået gennem dette projekt, vil være brugbar ved design af fremtidige vacciner og ved antistofbaserede lægemidler, der efterligner et naturligt immunrespons og som derfor er effektive med få bivirkninger. Resultaterne er desuden af fundamental immunologisk interesse, da konklusionerne fra dette projekt formentlig vil kunne overføres til en generel forståelse af sammensætningen og udviklingen af specifikke antistofrepertoirer.

Abstract

The human immune system is constantly working to protect the body against potentially harmful invading substances and organisms such as virus or bacteria (antigens). Antibodies are important molecules which are used by the immune system to recognize these invaders. Through the specific binding of antibodies to antigens, these are recognized as foreign and can then be eliminated by effector cells of the immune system. The antibody molecule consists of two parts. One is similar for most antibodies and is used to signal to the cells of the immune system to take action. The other part differs much between different antibodies. This is the part that binds to foreign antigens. In this fashion, the immune system targets almost anything foreign in the body by the generation of a diverse antibody repertoire. When the antigen is recognized repeatedly, the antibody repertoire matures and the binding strengths of the antibodies to antigens are increased facilitating a more efficient elimination.

The Symplex™ technology is the only means of isolating specific human antibody repertoires. During my PhD work, we have utilized this technology to isolate three consecutive antibody repertoires against tetanus from two donors with 1.5 to 4.5 year intervals. Additionally, we have isolated an antibody repertoire from a donor after receiving the very first tetanus vaccination in order to compare early and late repertoires. These seven repertoires are the most comprehensive specific antibody repertoires reported to date. Further, it is the only study that follows the development of antibody repertoires after several vaccinations of the same donors. Based on this large panel of antibodies, we have reported the first estimate on the size of specific antibody repertoires in humans. We have also – as the first – reported that antibody repertoires reach a ceiling in binding strength after several vaccinations. Additionally, we have found that the antibody response after the first encounter with antigen is similar to later responses. The ceiling in binding strength is, however, reached only after several vaccinations.

The understanding gained through this project will be of use in design of future vaccines and antibody therapeutics that mimic natural immune responses and hence are efficient with limited side effects. The results are of fundamental immunological interest as well since the conclusions regarding repertoire size and functional maturation limits of tetanus specific repertoires may well be applicable to antibody repertoires of other specificities than tetanus.

Contents

Original Papers

This thesis is based on the following papers which are referred to in the text by their numbers (1-3). The papers are appended at the end of the thesis.

Paper 1:

Poulsen, T. R., Meijer, P.-J., Jensen, A., Nielsen, L. S., Andersen, P. S.
Kinetic, Affinity, and Diversity Limits of Human Polyclonal Antibody Responses against Tetanus Toxoid
J. Immunol. 2007, 179: 3841–3850

Paper 2:

Poulsen, T. R., Jensen, A., Haurum, J., Andersen, P. S.
Limits for affinity maturation and repertoire diversification in developing human antibody responses
2009 (Manuscript)

Paper 3:

Poulsen, T. R., Andersen, P. S.
The role of genetic diversification mechanisms in antigen-induced antibody repertoires
2009 (Manuscript in preparation)

Abbreviations

A	adenine
aa	amino acid
Ab	antibody
Ag	antigen
AID	activation-induced cytidine deaminase
BCR	B cell receptor
C	constant domain
C	cytosine
C_H	constant, heavy chain
C_κ	constant, kappa chain
CDR	complementarity determining region
CMV	cytomegalovirus
CSR	class switch recombination
D	diversity
dsDNA	double-stranded DNA
Fab	fragment, antigen binding
FACS	fluorescence activated cell sorting
FW	framework
G	guanine
GC	germinal center
H	heavy
HC	heavy chain
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
Ig	immunoglobulin
IGHV	immunoglobulin heavy chain variable
IGKV	immunoglobulin kappa chain variable
IMGT	the international immunogenetics information system
J	joining
κ	kappa
K_D	affinity – dissociation constant (M)
k_{off}	off-rate (s^{-1})
k_{on}	on-rate ($M^{-1}*s^{-1}$)
L	light
LC	light chain
mAb	monoclonal antibody
pAb	polyclonal antibody
RA	rheumatoid arthritis
RAG1	recombination activating gene/protein 1
RAG2	recombination activating gene/protein 2
rpAb	recombinant polyclonal antibody
RSS	recombination signal sequence
RT-PCR	reverse transcription polymerase chain reaction

SHM	somatic hypermutation
SPR	surface plasmon resonance
ssDNA	single-stranded DNA
T	thymine
TdT	terminal deoxynucleotidyl transferase
U	uridine
UNG	uracil-DNA glycosylase
V	variable
V_H	variable domain of the heavy chain
V_K	variable domain of the kappa chain
VSV	vesicular stomatitis virus

1. Introduction

The immune system is a versatile defense system that has evolved to protect us from invading pathogenic bacteria and virus, and from toxins and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. The system has a remarkable specificity and is able to distinguish different pathogens. Once the foreign molecule has been recognized, a variety of cells and molecules are recruited to mount an effector response. In humans and other higher vertebrates, adaptive immunity ensures that later exposures to the same pathogen triggers a memory response characterized by a more rapid and heightened immune reaction than the first encounter. An important feature of immunity is the discrimination between the body's own cells and molecules and foreign molecules (self and non-self). Antibodies are some of the mediators of the specificity of the immune system since they are generated to bind specifically to a certain target. All the antibodies targeting one antigen make up a repertoire which can be altered and improved with multiple challenges by the process of affinity maturation in conjunction with selection mechanisms. With their dual function, antibodies act as powerful adapter molecules by binding specifically to foreign molecules with one end and then triggering immunological effector systems by the subsequent binding to receptors on cells or to other effector molecules. By the specific binding to e.g. viruses, toxins or bacteria the antibodies thus facilitate elimination of the foreign molecule from the host. However, recognition of a massive amount of different cells and molecules (antigens) requires a tight regulation of antibody recognition since the generation of widely recognizing antibodies also produces antibodies that bind to self. If not removed, these self-binding antibodies can result in numerous autoimmune diseases (e.g. systemic lupus erythematosus and rheumatoid arthritis) (Meffre et al. 2008). However, the amazing ability to generate both specificity and diversity is one of the hallmarks of adaptive immunity.

Owing to their great specificity, antibodies have been utilized in a wide variety of diagnostic applications for a long time, e.g. in pregnancy tests, blood type tests, as medical diagnostic tools etc. Also in the clinic, antibodies are widely used. They have been used for many years as therapeutics as plasma from individuals who have been challenged with different antigens naturally or by immunization. Since these preparations contain plasma from over 1000 individuals, they contain large amounts of polyclonal antibodies against many different antigens. Previously, these so-called gammaglobulin injections were usually given in an attempt to temporarily boost a

patient's immunity against certain diseases. In Europe it was common practice to recommend healthy people traveling to high risk countries in e.g. Africa and Asia these injections to protect against e.g. hepatitis A by passive immunization. However, this practice has largely been replaced by specific vaccines against the diseases in question, e.g. Havrix against hepatitis A. Nevertheless, immunoglobulins (Ig) from human donors are still used in the clinic as both gammaglobulin (bolus injections) and intravenous immunoglobulin (IVIg) to treat a number of acute and chronic infectious diseases, inflammatory and autoimmune disorders and to prepare patients for transplantations. Further, larger animals such as horses, goats and rabbits are used for raising antibodies against several toxins for passive immunization with so-called antiserum or antivenin.
In 1975, Cesar Milstein and Georges Köhler developed the hybridoma technology for recombinant production of monoclonal antibodies for specific targets (Köhler and Milstein 1975). This allowed for production of large quantities of antibodies targeting specific diseases. In the beginning, these antibodies were of murine origin; however when used in the clinic, there were problems because murine antibodies are recognized as foreign when administered in the human body resulting in less efficacy and unwanted immune or allergic reactions. Later, the recombinant antibodies were engineered to resemble human antibodies more. The so-called chimeric antibodies – antibody hybrids with human constant regions and mouse variable regions – were engineered in an attempt to overcome this problem. The chimeric antibodies were further improved by grafting only the antigen-contacting regions of the murine antibody into a human antibody which became known as humanized antibodies. Recent advancement in antibody technologies has enabled the isolation and recombinant production of truly human antibodies. The Symplex™ technology developed at Symphogen A/S is one technique that allows for the isolation of large repertoires of fully human antibodies from human donors. These antibodies can be combined into polyclonal compositions targeting several molecules simultaneously. This creates a higher antibody density on target molecules which enhances biological effector functions. In addition, polyclonal preparations are less sensitive to mutation of the antigen and can target several bacterial or virus strains in one preparation. In this way, recombinant polyclonal antibodies (rpAb) resemble plasma derived immunoglobulins. The big difference is that rpAb do not contain a large fraction of irrelevant antibodies, they can be produced in essentially unlimited quantities and importantly, the risk of transferring infections between individuals is eliminated.
The Symplex technology facilitates the isolation of extensive antibody repertoires against specific antigens while conserving the natural pairing of heavy and light chains in each antibody clone. This

unique feature ensures that the native affinity and specificity of each antibody is conserved. Further, the technology has a very high throughput compared to other antibody isolation techniques. The molecular and cellular basis for development of diverse and effective antibody repertoires remains only partly elucidated due to different limitations of previous antibody isolation techniques. The unique features of Symplex make the technology an excellent tool for studying diversity and development of specific human antibody repertoires. In the current study, we have utilized the ability of the Symplex technology to isolate large repertoires of antibodies against tetanus toxoid from human donors. This has allowed us to explore the field of specific human antibody repertoires from healthy donors in a fashion that has not been possible until recently.

During this project, we have studied comprehensive tetanus specific antibody repertories from two hyperimmune adults after each of three tetanus vaccinations. The isolation of these repertoires has allowed us to investigate the genetic and functional diversity of mature specific antibody repertoires (paper 1-3) and follow the development after several antigen encounters (paper 2 + 3). Further, we have isolated a primary repertoire from a naïve donor after receiving the very first vaccination (paper 3). This has allowed for a genetic study of antibodies generated in the early response. These seven specific antibody repertoires collected before and during my PhD work, comprise a total of 605 unique antibodies. This is by far the most comprehensive panel reported to this date of specific and naturally selected antibodies isolated directly from single human donors. Further, it is the only study that follows the development of specific antibody repertoires in the same donors. We have selected a subset of the antibodies for functional characterization by surface plasmon resonance (SPR). In total, affinities have been determined for 157 tetanus specific antibodies. This has allowed for the establishment of an experimentally determined affinity ceiling for antibodies selected naturally (paper 2). It has also allowed for a study on the influence of antibody diversification on antigen binding (paper 1+ 3). Tetanus toxoid was chosen as antigen for these studies because it is one of the most potent vaccines in clinical use and it is safe. Further, it is a structurally complex antigen ensuring that the studied antibody repertoires are not restricted by the antigen itself rather than *in vivo* selection mechanisms. We anticipate that the results concentrating on antibody repertoires against tetanus toxoid can be transferred to a more general understanding of antibody affinity ceilings and antibody repertoire development.

In the following, antibody structure and generation will be described. Further, antibody diversification mechanisms will be described as well as the generation and selection of specific antibody repertoires and affinity maturation. These subjects will be related to our own findings

during my PhD project. These include antibody affinity limits, genetic development of antibody repertoires and diversification of antibodies by e.g. somatic hypermutations and deletions of codons. Finally, further work that builds on the project results will be suggested.

2. Antibody structure[1]

Antibodies are glycoproteins consisting of four immunoglobulin polypeptide chains with a basic composition of two identical light chains of molecular weights of about 25 kDa each and two identical heavy chains, weighing about 50 kDa each. Each light chain is linked to one heavy chain by a disulfide bond and the two heavy chains are further connected to each other by disulfide bonds. The overall structure of an antibody resembles the letter Y or T (see figure 1).

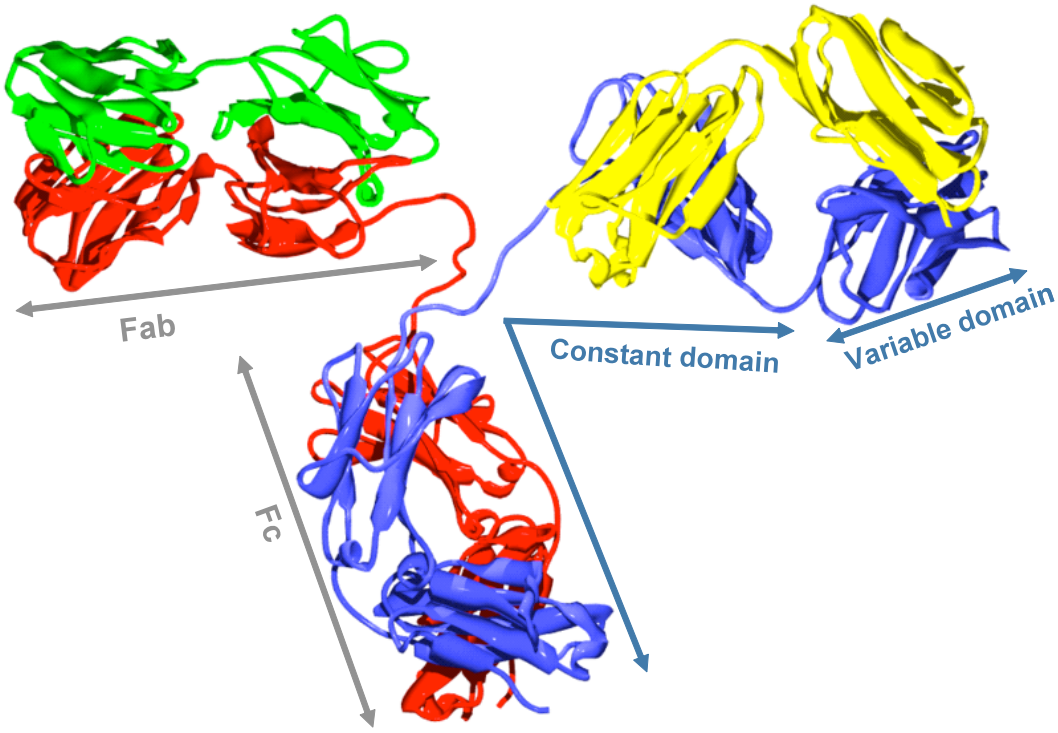

Figure 1. General structure of an antibody molecule.

The identical heavy chains are shown in red and blue while identical light chains are shown in green and yellow (modified from http://en.wikipedia.org/wiki/Antibody).

The light chains each consists of two globular domains – immunoglobulin folds constituted by two β sheets stabilized by hydrophobic interactions and disulfide bonds. Each heavy chain contains 4-5 of these globular domains (depending on heavy chain isotype). Each chain has an amino-terminal variable (V) region, which differs significantly for different antibodies whereas the rest of the

[1] This section is written based on sections from "Immunology" by Richard A. Goldsby et al. 5th edition and "Immunobiology" by Charles A. Janeway et al. 5th edition

molecule consists of so-called constant (C) regions. It is the genetic variability of the V gene that determines the antigen recognition diversity (Honjo and Habu 1985). The V domain contacts the antigen while the C regions mediate physiological/effector functions like e.g. interaction with effector cells or complement. The greatest sequence variation of the V domains is concentrated in a few discrete regions which are the parts of the sequence that correspond to the loops that join the β strands. These regions are called complementarity determining regions (CDRs) because they form the antigen binding site – or paratope – of the antibody. The paratope contacts the antibody-binding site – or epitope – on the antigen. Depending on the structure and size of the antigen, it is contacted by one or several CDRs. There are three CDRs in the V domain of each heavy and each light chain (termed CDR1, CDR2, and CDR3) and the CDR3 regions exhibit more variation than CDR1 and CDR2 (Padlan 1994, Davies and Cohen 1996). The CDR3 of the heavy chain is generally regarded as the major contributor to the antigen-specificity. The parts of the variable domains that display less variation are called framework regions (FWs) and serve as scaffolds for the CDR loops.
There are five antibody isotypes which are determined by the heavy chain class: immunoglobulin A (IgA, α), IgD (δ), IgE (ε), IgG (γ), and IgM (μ). Furthermore, there are two subtypes of the IgA class (IgA1 and IgA2) and four subtypes of the IgG class (IgG1-4) in humans. The different isotypes are structurally distinct and exert different biological functions. For example, IgG – the most abundant class in serum – is secreted as a monomer while IgM is secreted as a pentamer and hence exert a high avidity for antigen. Of differences in biological functions can be mentioned that for instance IgG2a is a potent activator of the complement system (Benhnia 2009).
In contrast to the many heavy chain isotypes, there are only two light chain types; lambda, λ, and kappa, κ, with a frequency of 40% and 60%, respectively, in humans (de Wildt 1999a). One B cell clone can express one heavy chain subclass at one time point but the subclass can be changed over time (class switching). Similarly, each B cell clone can only express one type of light chain (either κ or λ) at one time point. The class of light chain can however be changed by the process of receptor editing (only from κ to λ).
When monomeric antibodies are digested with papain (an experiment first conducted by Rodney Porter), the heavy chains are cleaved in the hinge region producing a fragment with receptor-binding activity – the Fc fragment – and two identical Fab fragments with preserved antigen-binding activity (see figure 1) (Porter 1991s). In many assays, only this antigen-binding part consisting of the variable domain and the first constant globular domain of each heavy (C_H1) and light chain (C_κ) of the antibody is used in order to have a monovalent binder. For example, when

measuring binding affinity of the antibody-antigen complex by surface plasmon resonance (SPR), it is very convenient to use Fab fragments in solution since multivalent molecules “wander” on the antigen surface resulting in a higher measured affinity.
Antibodies can either be expressed as membrane-bound antibody which is transported to the B cell membrane and functions as a B cell receptor (BCR) or they can be secreted in a soluble form. While the latter has a hydrophilic amino acid (aa) sequence at the C-terminal end, the BCR is expressed with a hydrophiobic transmembrane sequence and a short cytoplasmic tail. Whilst the secreted antibodies can exert different effector functions by binding of their Fc ends, the BCR plays a role in signaling to the B cell, e.g. when detecting antigen.

2.1 Affinity of antibodies

The affinity of an antibody is a measurement of how well the epitope fits in the antigen binding site of the antibody; in other words, the affinity is the binding strength of the antibody-antigen interaction. The affinity is measured as the steady state of the association and dissociation of antibody and the antigen it binds to:

$$Ag + Ab \leftrightarrow Ag\text{-}Ab$$

The affinity is defined by the rate at which the Fab fragment and antigen dissociate divided by the rate at which they associate:

$$K_D = \frac{[Ag][Ab]}{[Ag - Ab]} = \frac{k_{off}}{k_{on}}$$

Affinities can be measured by either measuring the concentrations of bound and unbound Fab fragment and antigen (Ag) at different time points, or the association and dissociation rates can be measured in real time.

3. The Symplex™ Technology

During the last couple of decades, many technologies have been developed for the cloning of antibodies from humans or mice to be able to study the immune system or to isolate monoclonal antibodies. For many years, larger panels of antibodies were isolated successfully by different combinatorial library technologies such as phage and yeast display (Smith 1985, McCafferty et al. 1990, Clackson et al. 1991, Boder and Wittrup 1997), and recently mammalian display (Beerli et al. 2008). These technologies have allowed for isolation of antibodies against virtually any antigen (Marks et al. 1991). Moreover, smaller panels of antibodies with cognate pairing of heavy and light chain have been isolated by the elaborate hybridoma technology (Kohler and Milstein 1975). However, these techniques have low efficiency or scramble heavy and light chains of single antibodies and are thus not suited for the isolation of larger repertoires of antibodies of defined specificities selected *in vivo* (Stahli et al. 1980, Babcook et al. 1996). In recent years, a number of technologies have been developed that are efficient with cognate pairing of the heavy and light chains of each antibody (Tiller et al. 2008, Wardemann et al. 2003, Traggiai et al. 2004, Lanzavecchia et al. 2007, Jiang et al. 2006, Wrammert et al. 2008, Love et al. 2006).
The Symplex technology is a proprietary technology developed at Symphogen A/S which facilitates rapid isolation and cloning of extensive antibody repertoires from human blood samples. This allows for capture of original antibody repertoires against specific antigens such as viruses, toxins, or bacteria. The technology conserves the natural pairing of heavy and light chains in each antibody clone. This unique feature ensures that the native affinity and specificity of each antibody is conserved. Further, the technology has a very high throughput compared to most antibody isolation techniques. These features make the Symplex technology ideal for studying human antibody repertoires in detail and for isolation of therapeutic antibodies. The technology is currently being used in the development of fully human recombinant polyclonal treatments and has been used for the isolation of antibody repertoires against complex antigens including influenza virus, tetanus, respiratory syncytial virus (RSV), cancer, Vaccinia virus (smallpox), hepatitis B virus and some non-disclosed bacterial targets.

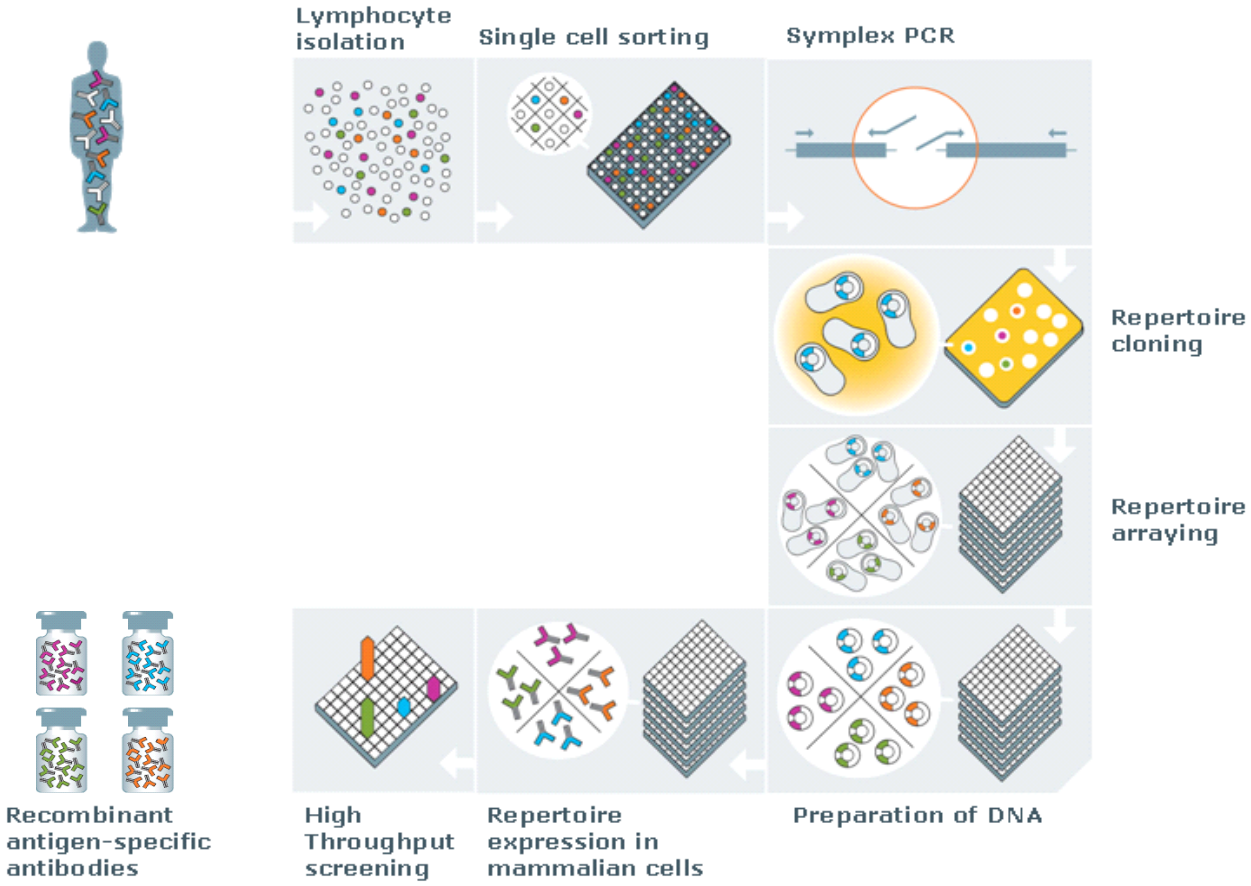

Figure 2. Symplex technology overview. *Printed with permission from Symphogen A/S.*

Figure 2 shows an overview of the steps in the Symplex technology. In short, the technology is carried out by recovering the lymphocyte fraction from blood from a donor who has been challenged with an antigen either naturally or by immunization within the last 5-9 days (preferably on day 6) (Meijer et al. 2006). This allows for the capture of recently activated plasma blasts migrating to secondary lymphoid tissues (Odendahl et al. 2005). The plasma blasts are then isolated from the B cell fraction by fluorescence activated cell sorting (FACS). The plasma blasts are single cell sorted into 384 well plates containing buffer and primers and a reverse transcription polymerase chain reaction (RT-PCR) is performed directly in the plates. The RT-PCR is designed to link C_H1-V_H genes to κ light chain encoding genes (C_κ); see figure 3: Symplex PCR. Hereafter, the pool of C_H-V_H-C_κ gene products is inserted into an expression vector. The linker sequence is replaced by a bidirectional promoter to allow Fab or antibody expression (depending on the expression vector) followed by the insertion of the vector into *E. coli* or mammal cells such as CHO or HEK. Finally, the clones are screened for specificity against the antigen in question.

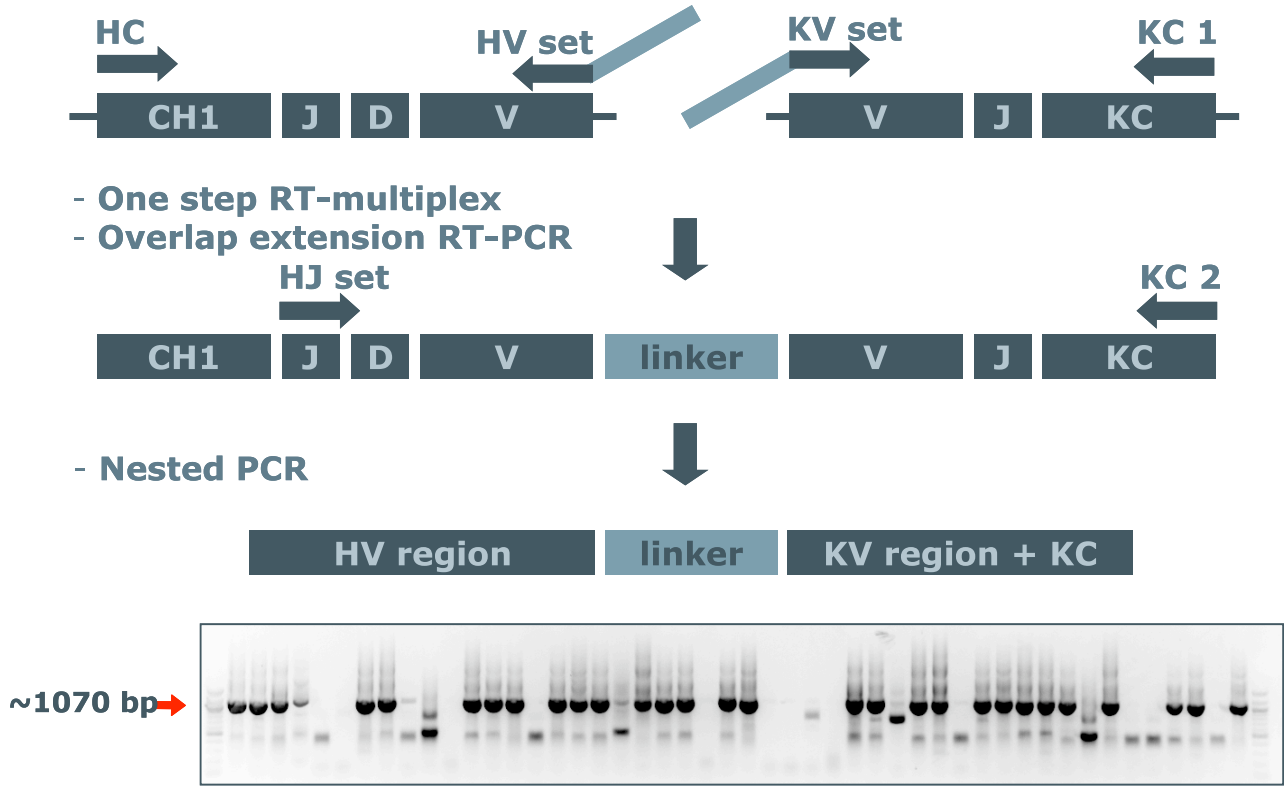

Figure 3. Symplex PCR overview.

The Symplex polymerase chain reaction takes place in two steps: First, a reverse transcription polymerase chain reaction (RT-PCR) is performed which links C_H1-V_H genes to κ light chain encoding genes ($C_κ$). Second,a nested PCR is performed to amplify the heavy chain variable regions linked to kappa light chain genes. The two consecutive PCR reactions result in DNA pieces of ~1070 bp which can be checked by agarose gel electrophoresis. Printed with permission from Symphogen A/S.

The Symplex technology offers a unique opportunity to study the development of human antibody repertoires which has not been possible until recently. During this project we have used the technology for the isolation and characterization of human antibody repertoires against tetanus toxoid from two hyperimmune donors and a tetanus-naïve donor (paper 1, 2 and 3). Each of the two hyperimmune donors were boosted with the tetanus vaccine 3 times separated by 3,5 years for the first and second boost and by 1.5 years for the second and third boost. Antibody repertoires were isolated after each boost and screened for tetanus binding. A number of positive hits were sequenced (see paper 1, 2 and 3 for details) resulting in 6 tetanus specific repertoires each consisting of 82-176 antibodies from each of the two hyperimmune donors. A repertoire of 11 antibodies was isolated from the previously naïve donor.

4. Antibody generation and diversification of antibody repertories

Antibody genes are generated during the development of B lymphocytes. The massive diversity of the antibody repertoire is generated by two mechanisms. The first mechanism being recombination of immunoglobulin gene variable (V), diversity (D), and joining (J) gene segments (V(D)J recombination) during the early stages of B cell development in the bone marrow. Heavy chains are generated by combination of a V, D and J segment, whereas light chains only have recombined V and J segments (Joho et al. 1980, Cook and Tomlinson 1995, Matsuda and Honjo 1996). The second mechanism that occurs within secondary lymphoid organs is somatic hypermutation (SHM) of functional immunoglobulin genes from antigen-activated B cells. The diversity generated by these two mechanisms not only provides protective humoral immunity but also generates potentially harmful clones expressing antibodies that bind to self-molecules (autoantibodies) (Clarke et al. 1985, Jacob and Kelsoe 1991). Therefore developing B lymphocytes undergo both positive and negative selection. These processes ensure that the antibody producing B cells produce functional immunoglobulin and remove autoreactive B cells at central and peripheral B cell tolerance checkpoints (Meffre and Wardemann 2008). Apart from clonal deletion, autoreacting B cells can maintain tolerance to self antigens by the mechanisms of receptor editing and anergy (Casellas et al. 2001).

4.1 V(D)J recombination

The process of V(D)J recombination combines and assembles antibody genes from a pool of gene segments (germline genes) in developing B lymphocytes (Tonegawa 1983). These gene segments can not be transcribed and translated into complete protein chains until they are rearranged into functional genes. The site-specific rearrangement process creates an immense diversity of antibodies at the molecular level. Although, the Ig genes are generated by a relatively small number of gene segments, the pre-immune diversity based on these combinatorial reactions alone can generate over 10^7 different antibody specificities (in theory) (Papavasiliou and Schatz 2002). The genes encoding the sequence-specific DNA endonuclease initiating V(D)J recombination (McBlane et al. 1995, van et al. 1995) are recombination activating gene 1 and 2, or simply RAG1 and RAG2. These two genes were discovered by David Schatz, Marjorie Oettinger, and David Baltimore at MIT during the late 1980's. They also discovered that these two genes reside only a few kilobases from each other (Schatz et al. 1989, Oettinger et al. 1990). Further experiments demonstrated that

RAG1 and RAG2 are almost invariably expressed together and that their coexpression results in potent activation of V(D)J recombination (Oettinger et al. 1990). Both genes are required for the introduction of double-strand breaks initiating the recombination process (Oettinger et al. 1990). The breaks are introduced between V, D, and J coding segments and their flanking recombination signal sequences (RSSs) consisting of conserved heptameric (consensus, 5'-CACAGTG) and nonameric (consensus, 5'-ACAAAAACC) motifs connected by 23 or 12 bp variable sequence (Max and Leder 1979, Sakano et al. 1979). The cleavage occurs in two steps in ordered sequence: nicking and hairpin formation (McBlane et al. 1995, Lewis 1994). A nick is first introduced in the top strand 5' of the RSS heptamer at the junction between the heptamer and the V, D or J coding segment. This exposes a 3' hydroxyl group at the end of the coding flank and a 5' phosphate group attached to the heptamer end. The 3' hydroxyl group attacks the antiparallel strand and cleaves the DNA which is followed by the formation of a hairpin coding end and a blunt 5' phosphorylated signal end (van Gent et al. 1996). When both participating DNA substrates have been cleaved, the RSS ends are precisely joined and excised as a circular product. Simultaneously, the coding ends are modified and joined imprecisely in a process involving non-templated (N) and templated (P) nucleotide addition in the non-homologous end-joining DNA repair pathway (reviewed in references (Bassing et al. 2002, Gellert 2002, Fugmann et al. 2000)). This junctional flexibility leads to both productive and non-productive rearrangements by e.g. out of frame sequences resulting in stop codons or the introduction of amino acids that "disturb" proper folding of the antibody.

4.2 Further diversification of antibodies

After B lymphocytes leave the bone marrow following successful generation of functional antibodies, they undergo two further receptor diversification processes upon encounter with antigen: somatic hypermutation (Berek and Milstein 1988) and class switch recombination (CSR) (Sakano et al. 1980, Stavnezer 1996). The first process is initiated by the binding of antigen and introduces point mutations in the variable region of immunoglobulin genes while the latter changes the constant region to other constant regions (isotypes) producing antibodies of the same specificity but with other effector functions (Besmer et al. 2004). Both of these processes are executed by the enzyme activation-induced cytidine deaminase (AID) (Besmer et al. 2004) and are generally tightly controlled to occur in germinal centers (GCs) only (Odegard and Schatz 2006).

4.2.1 Somatic hypermutation

Somatic hypermutation introduces additional diversity to that introduced by combinatorial rearrangement of V(D)J gene segments and by the association of different heavy and light chains. Somatic hypermutation is initiated by the binding of the B cell receptor to antigen and introduces point mutations into the variable region of immunoglobulin genes (Besmer et al. 2004). AID is required for the generation of somatic mutations together with some co-factors (Muramatsu et al. 2000, Odegard and Schatz 2006, Wilson et al. 2005, Neuberger et al. 2003). Naturally, effects of this protein have been studied intensively during the last few years (Odegard and Schatz 2006, Besmer et al. 2004, Muramatsu et al. 2000). The expression of AID is generally confined to GC B cells (Odegard and Schatz 2006) and is tightly regulated. The protein deaminates single-stranded DNA (ssDNA), but not RNA or double-stranded DNA (dsDNA). And it has a preference for deaminating cytidines in SHM hotspot motifs (such as (A/G/T)G(C/T)(A/T) or (A/T)(A/G)C(T/C/A)) (reviewed by (Odegard and Schatz 2006)). The deamination happens during transcription when AID can act on small regions of ssDNA on the non-transcribed strand. AID initiates SHM by deamination of C nucleotides, and the resulting U-G (uridine-guanosine) mismatch can lead to mutation in several ways (Neuberger et al. 2003). If the mismatch is not repaired before DNA replication, DNA polymerases insert A nucleotides opposite U which lead to transition mutations. However, if uracil-DNA glycosylase (UNG) removes the U, replication can give rise to both transitions and transversions through an error-prone mismatch repair machinery (Wilson et al. 2005, Odegard and Schatz 2006). This machinery is also thought to create mutations at A-T sites near the initial mismatch (Neuberger et al. 2003).

SHM are introduced at a rate of app. 10^{-3} mutations per base pair per cell division, which is 10^{6}-fold higher than the spontaneous mutation rate in somatic cells (Odegard and Schatz 2006). Hence, SHM are tightly controlled and targeted specifically at variable regions of Ig genes. As described above, the mutations exhibit preferences for transitions and are not distributed randomly over the entire length of the variable regions of antibodies. The hotspots where mutations are often found are generally structurally situated at the edge of a loop in the antibody structure (Sale et al. 2001) probably because this is where they are best tolerated. Further, SHM decrease exponentially with distance from the transcription start site (Rada and Milstein 2001). By studying hapten responses, it has been demonstrated that the average number of somatic hypermutation per antibody increases with several antigenic challenges (Berek and Milstein 1987). In paper 2, we demonstrate that the number of SHM per antibody varies between 0 and 40 on amino acid levels for hyperimmune

responses against tetanus toxoid with 50% of the antibodies having between 16 and 25 SHM. We further demonstrate that the number of hypermutations in specific mature and saturated antibody repertoires is rather constant after several immunizations but can differ slightly between individuals. This feature might reflect that antibodies lose the typical Ig structure when too many SHM are introduced and hence it cannot bind the antigen or initiate proper effector functions. In paper 2, we also demonstrate that somatic hypermutations in mature specific antibody repertoires follow a normal distribution in agreement with a random system. It follows from the stochastic nature of single somatic hypermutations that they can be deleterious to the cell (either by the creation of a non-functional or self-recognizing immunoglobulin), increase or decrease binding affinity or be neutral depending on the type and the location of the substitution. Hence, it is conceivable that there is a general maximum to the number of tolerated SHM.

In order to investigate the level of hypermutation in early responses, in paper 3, we isolated a primary anti-tetanus repertoire. Surprisingly, the 11 antibodies isolated from this previously naïve donor on day 6 after the first tetanus vaccination displayed an average of 15 SHM per heavy and light (H+L) chain pair. The antibodies each had between 6-22 SHM, indicating that hypermutations can occur relatively fast in primary repertoires and much faster than what has been observed in hapten responses (Berek and Milstein 1987).

It is known that the quality of the antibody response decreases with age, with a lower level of specific antibodies and an increased level of non-specific antibodies generated in response to vaccination (Howard et al. 2006). Lately, it has been demonstrated that the average number of somatic hypermutations per antibody is significantly raised in the elderly (>75 years) compared to non-elderly (Gibson et al. 2009, Chong et al. 2003). Banerjee et al. (Banerjee et al. 2002) have shown that the somatic hypermutation process occurs at the same rate in young and old humans. Therefore, it is suggested that the observed increase might be the result of an immense accumulation in SHM over many years in combination with a waning amount of bone marrow and it could be partly responsible for a less effective immune system in the elderly (Banerjee et al. 2002). Another possibility could be a less tight selection on antibodies with age.

There are several opinions about the importance of somatic hypermutation for the *in vivo* generation of a high-affinity antibody response. Manivel et al. (Manivel et al. 2000) have proposed that non-mutated antibodies are more flexible and hence cross-reactive whereas those that have introduced mutations are more rigid. So combined with selection, somatic mutations yield antibodies of high specificity and high affinity (a lock and key mechanism) compared to germline antibodies.

A perhaps more controversial theory for the role of SHM has been proposed by Andersson et al. (Andersson et al. 1998). They argue that the SHM apparatus originated in the Peyers patches in the GI tract in response to gut bacteria where it is important because of antigen-independent antibody production. The apparatus was then introduced into GCs as these developed. Because of the selection of B cell clones by T cells, the SHM apparatus is not essential in GCs but does not hurt either as long as B cell clones are actively selected based on affinity. It is, however, discussable whether such an "expensive" feature on the cellular level as somatic hypermutations would exist if it were expendable. In paper 3, we argue that SHM are not directly correlated to affinity or binding kinetics although they do yield antibodies of higher affinity and better binding kinetics compared to germline "ancestor" antibody clones on two occasions. Thus, somatic hypermutation in itself does not ensure higher affinity clones but combined with selection for these it is an indispensible feature in the generation of efficient and diverse antibody repertoires of high quality.

4.2.2 Insertions and deletions

In addition to the structural diversity introduced by the recombination of different gene segments and the generation of somatic hypermutations, entire codons can be inserted or deleted in rearranged antibody genes. These insertions and deletions are clustered at hot-spots in the antigen binding site and they often result in the generation of new combinations of canonical loop structures or entirely new loops creating an expanded antibody structure space (de Wildt et al. 1999b). The insertions and deletions involve repetitive sequence motifs in the antibody-encoding genes, and it has been suggested that they occur through polymerase slippage (Lantto and Ohlin 2002). 1.5–9.7% of normal B cells have been reported to carry insertions or deletions in their hypermutated antibody-encoding genes (de Wildt et al. 1999b, Goossens et al. 1998, Ohlin and Borrebaeck 1998, Wilson et al. 1998, Reason and Zhou 2006). The actual frequency may be even higher, since insertions and deletions in the CDR3 of the heavy chain and at the end of the CDR3 of the light chain are usually unidentifiable due to the unknown length of the primary transcripts (Lantto and Ohlin 2002). It has been demonstrated that IGHV genes specifically carry repetitive trinucleotide motifs in CDR1 and CDR2, thus making these parts of the genes that encode highly flexible structures particularly prone to functional deletions (Lantto and Ohlin 2002). Like the hypermutations, many of the modifications result in an inability of the antibody gene to encode a functional protein, either because the deletions and insertions produce an out-of-frame gene, or because the modification alters the protein so dramatically that it cannot fold properly. Recently, Reason and Zhou (Reason

and Zhou 2006) have demonstrated by clonal lineage analysis that insertion and deletion events occur throughout the course of the somatic maturation of individual antibody clones. The co-occurrence of these modifications with base-pair substitutions that appear to have arisen from SHM suggests that insertions and deletions may be a normal consequence of the somatic maturation of antibody responses. Hence, it also suggests that these processes contribute to diversification of antibody repertoires (Ohlin and Borrebaeck 1998).

In paper 3, we demonstrate that deletions are found in mature specific antibody repertoires against tetanus. We found that the process had occurred in 6.4% of the clonotypes isolated (based on analysis of V segments only) with 3 in 5 found in heavy chains. No insertions were observed in our rather large data sets. Most of the deletions were found close to CDR1s or CDR2s in both heavy and light chains supporting the findings by de Wildt et al. (de Wildt et al. 1999b) that they often result in the generation of new combinations of canonical loop structures or entirely new loops which are then likely to contact the antigen directly. The frequencies of insertions and deletions may, however, be underestimated since insertions and deletions in CDR3 regions are difficult to assign. Nevertheless, our results demonstrate that deletions are indeed found in mature specific antibody repertoires against tetanus. However, the small frequency by which they occur – and the lack of insertions – suggest that they are not a dominant means of diversification.

4.2.3 Class switch recombination (CSR)

After activation of a mature B cell that has recognized antigen, certain cytokines can stimulate the B cell to switch heavy chain class while keeping the antigenic specificity by the process of class-switch recombination (CSR) or isotype switching (Sakano et al. 1980, Stavnezer 1996). This can alter the antibody's effector function. It is done by recombination of the already joined V-D-J segment of the variable domain of the heavy chain with a C_H gene segment of another heavy chain class, e.g. the μ heavy chain can be switched to γ (IgM⇒IgG). The B cell switches the isotype in a certain sequence (μ, δ, γ, ε, α) and can never go back to a previously used isotype since the used gene segments are excised from the genome during recombination. The enzyme AID is required for CSR as well as for somatic hypermutation (Revy et al. 2000).

4.2.4 Receptor editing

Receptor editing (sometimes referred to as receptor revision) is thought to be a process by which self-reacting antibodies escape deletion and it has been described as a dominant mechanism controlling central B cell tolerance in addition to clonal deletion and anergy (Pelanda and Torres

2006, Ait-Azzouzene et al. 2005). The mechanism was identified by two groups in the early 1990's (Gay et al. 1993, Tiegs et al. 1993) who noticed that a small proportion of immature B cells expressing auto-reactive immunoglobulin were not submitted to clonal deletion. Rather, the auto-reactivity of the B cell clone was eliminated through secondary V(D)J recombination. Hence, when the immature B cell binds a self-antigen, maturation can be arrested while the cell upregulates expression of RAG1 and RAG2. This in turn initiates further rearrangement of the V(D)J gene segment DNA that has not already been excised from the genome resulting in antibodies with new specificities. The process is relatively fast and has been shown to occur within about 6 hours in transgenic mice (Hippen et al. 2005). Hence, the B cell gets a second chance (or more) to produce a functional, non-self-binding antibody. In addition to rescuing self-reactive B cells, the process is thought to rescue some B cells with non-productive V(D)J rearrangements (Reth et al. 1986b, Reth et al. 1986a).

The process has been demonstrated to work on both immunoglobulin heavy and light chains (Gay et al. 1993, Tiegs et al. 1993, Papavasiliou et al. 1997, Nemazee and Weigert 2000). However, it has later been suggested that receptor editing of the heavy chain is an infrequent event, leaving light chain editing as the main mode of avoiding autoreactive specificities *in vivo* (Nadel et al. 1998b). In addition to rescuing self-reactive or non-functional B cells from deletion, receptor editing has been proposed as a process which serves to further diversify antibody repertoires (Radic and Zouali 1996, Pelanda et al. 1997, Ohlin and Zouali 2003). Groups at Rockefeller University, University of Cologne, and Scripps Research Institute have found that about 25% of all antibody molecules are produced by gene replacement (receptor editing). They thereby suggest that receptor editing actually represents a major force in shaping the antibody repertoire (Casellas et al. 2001). By contributing to the diversity of a given antibody repertoire, receptor editing may not only be a means of ensuring central tolerance but it may also contribute to affinity maturation by generation of clones with potentially higher affinity for antigen. This is also suggested by de Wildt et al. who have demonstrated that receptor editing occurs in the periphery as well as centrally, and that the same antibody lineage can be subjected to both receptor editing and somatic mutation (de Wildt et al. 1999a). In paper 3, we report that we do not observe cases of receptor editing in our relatively large data sets of tetanus specific antibodies. This suggests that receptor editing is not a major contributor to diversification of "normal" (non-autoimmune) antibody repertoires.

Although, receptor editing may be a dominant mechanism controlling central B cell tolerance and may be involved in affinity maturation (de Wildt et al. 1999a), the process itself needs to be tightly

controlled. This is supported by a study in patients suffering from rheumatic autoimmune diseases by Zouali (Zouali 2008). The study demonstrates that receptor editing is either impaired or accelerated in these patients. Notably, both alterations can result in pathogenesis since impaired secondary rearrangements might result in ineffective silencing of B cells, while exacerbation of receptor editing can give rise to autoreactive receptors from B cell clones that were initially devoid of autoreactivity (Zouali 2008).

5. Antibody repertoires

The study of antibody repertoires provides insights into the generation of antibody diversity as well as selection mechanisms or selection criteria. Therefore antibody repertoire characterization may help reveal the rules guarding antibody selection.

The imprecise joining of V(D)J segments can be estimated to generate a potential naïve repertoire of up to 10^{10} possibilities. However, since the available number of B cells is well below the number of variants that can be generated and some cells might generate the same recombination by chance, the available repertoire is several orders smaller – likely to be well below 10^8 (Berek and Milstein 1988). This also follows from the estimate that there are a total of about 10^8 B cells per person (Gibson et al. 2009).

In 1951, Jerne stated that the quantity and quality of antibodies changed during the course of the primary antibody response. He further explained that later during the primary response, antigen-specific antibodies exhibit improved affinity for the antigen and have switched isotype from IgM to IgG, IgE, or IgA (Jerne 1955). The statement was elaborated in the clonal selection theory which was proposed by Burnet in 1957. This theory states that initial antigen challenge is anticipated to lead to clonal expansion of the B cells that produce a cognate antibody specificity (M.F.Burnet 1959). The same theory anticipated that the random collection of Igs would include the generation of auto-reactive specificities, which would require silencing by the immune system.

The point mutations that are introduced into the Ig genes upon binding to antigen result in some of the altered antibodies having a higher affinity for the antigen in question. Because of the active selection during affinity maturation, cells containing these higher affinity antibodies will proliferate and survive preferentially. This is the background for selection of antibodies into specific repertoires from the non-specific and for the development of specific repertoires after several antigenic challenges.

5.1 Germline gene repertoire

During the past 30 years several groups have worked on the mapping and sequencing of the human antibody gene repertoire (Cook and Tomlinson 1995, Corbett et al. 1997, Matsuda et al. 1998, Ravetch et al. 1981, Schable and Zachau 1993, Tomlinson et al. 1992, Williams et al. 1996, Hieter et al. 1982, Kawasaki et al. 1997). The recombination of these gene segments generates antibodies that constitute the so-called naïve repertoire until they are hypermutated upon encounter with

antigen (Berek and Milstein 1988). All the information on antibody gene segments has been compiled by Lefranc and Lefranc, is available online where it is continually updated (IMGT Home Page 2009).

The heavy chain genes are located on chromosome 14. There are 53 functional V_H gene segments[2] (and 4 non-functional gene segments) divided into 7 families or subgroups (IGVH1-7) based on sequence homology. Each family shares at least 75% identity at the nucleotide level. The different families vary in size with the largest group being IGVH3 with 24 functional members (and 3 non-functional) while the smallest families IGVH2, IGVH5, IGVH6 and IGVH7 have between 1 and 3 members each. Located downstream from the V_H gene segments are 32 D gene segments belonging to 7 families (IGHD1-7), followed by 6 J_H gene segments which constitute one family each (IGHJ1-6). The light chain genes loci, encoding kappa (κ) and lambda (λ), are located on chromosome 2 and 22, respectively. There are 46 V_κ gene segments divided into 5 (IGKV1-5) families and 40 V_λ gene segments have been classified into 11 subgroups (IGLV1-11). IGKV1 and IGLV3 are the largest families with 22 and 11 members, respectively. There are 5 J_κ and 7 J_λ gene segments each representing one family. Normal human antibody repertoires usually consist of 60% antibodies with κ- light chains and 40% with λ-light chains (a 3:2 ratio) (de Wildt et al. 1999a). Besides the number of functional gene segments, there are also a number of non-functional genes or pseudogenes. Some of the structures generated by recombination of gene segments are deleterious to the cell because of frame shifts resulting in stop codons or the presence of non-tolerated amino acids in certain positions.

The sizes of the different gene segment families are roughly reflected in the usage of each family in specific antibody repertoires which leads to differences in the overall representation of the different genes (Brezinschek et al. 1995, Brezinschek et al. 1997, Huang et al. 1996, Brezinschek et al. 1998, Poulsen et al. 2007). Yet, within the families there are biases for usage of certain members. Skewed usage of gene segments and heavy-light chain combinations have been reported in non-specific antibody repertoires by several groups (Demaison et al. 1995, Tian et al. 2007, Cox et al. 1994, Stewart et al. 1993, Brezinschek et al. 1997, Foster et al. 1997). However, diverse germline repertoires are important in particularly primary infections since the initial binding of antigen to the B cell receptor – although it might be of low affinity – triggers further affinity maturation. Therefore, these biases might be the result of affinity maturation in combination with intrinsic

[2] All gene segment numbers are from IMGT®, the international ImMunoGeneTics information system® http://www.imgt.org (founder and director: Marie-Paule Lefranc, Montpellier, France).

genetic features causing preferential usage of certain segments such as chromosomal location (Williams et al. 2001) or variations in RSS (Nadel et al. 1998a).
Some groups have observed that germline antibodies (non-hypermutated antibodies) have more flexible paratopes than their hypermutated variants (Manivel et al. 2000, Brooks et al. 2008, Thomson et al. 2008). They hence exhibit a high degree of cross-reactivity (especially at physiological temperature) and the binding of germline and mutated antibodies to antigen have been found to be differentially affected by temperature increases (Manivel et al. 2000). Manivel et al. find that both types have increased dissociation at higher temperatures (Manivel et al. 2000). However, they find that primary repertoire antibodies have a decrease in association at higher temperatures while secondary repertoire antibodies increase association rates facilitating a qualitative distinction in on-rates. Zhou et al. actually suggest that polyreactive antibodies are a major contributor to the broad antibacterial activity of the natural (germline or non-hypermutated antibodies) antibody repertoire (Zhou et al. 2007).

5.2 Specific antibody repertoires

Many of the first studies on antibody repertoires were done on antibodies against different haptens (e.g. 2-phenyloxazolone, oxazolone, and nitrophenyl) in mice or rabbits. These repertoires turned out to be very restricted in terms of gene segment usage and were shown to be converging against very few clonotypes – especially after several challenges (Kaartinen et al. 1983, Takahashi et al. 1998, Berek and Milstein 1988). Berek and Milstein (Berek and Milstein 1988) have reported that the secondary and later responses to oxazolone in mice are dominated by a certain combination of gene segments and antibodies have certain hypermutations which are rarely found in the primary repertoire. They refer to this as a repertoire shift. However, the relevance of anti-hapten repertoires compared to challenges with more “natural” antigens is discussable. Hapten binding studies may not be relevant to microbial protein responses because the small size of haptens allows for high-affinity interactions with only a limited portion of the antibody-contacting surface (Persson and Ohlin 2007). Few studies have sought to determine how naturally occurring somatic mutations in human antibody genes affect the response to an important biologically relevant protein (Kallewaard et al. 2008). Nevertheless, more natural antigens have indeed proved to induce specific antibody repertoires dominated by a few clones (mono- or oligoclonal repertoires) such as antibody responses against Rhesus D (Andersen et al. 2006), human cytomegalovirus (HCMV) (Thomson et al. 2008), influenza virus (vaccine) (Wrammert et al. 2008), rotavirus (Kallewaard et al. 2008) and

Haemophilus influenzae type B (Hib) (Barington et al. 1996, Insel et al. 1985, Silverman and Lucas 1991). Interestingly, certain ethnic groups such as Navajos, Apaches and Alaskan Eskimos show an increased susceptibility for the latter where antibody responses are normally pauciclonal and dominated by antibodies using the Vκ A2 gene. Feeney et al. (Feeney et al. 1996) have demonstrated that these ethnic groups have a high frequency of polymorphisms in the Vκ A2 gene leading to defective rearrangements of this gene segment. Thus, the germline gene segments play an important role in the immunological control of certain pathogens especially where "normal" specific repertoires are dominated by a few clones.

In contrast to the restricted specific repertoires described above, antibody repertoires against other pathogens have been shown to be very diverse in terms of both number of clonotypes and hypermutations. These repertoires include responses against viruses and bacteria such as Vaccinia (Benhnia et al. 2009), cytomegalovirus (CMV) (Speckner et al. 1999, Ohlin et al. 1993), vesicular stomatitis virus (VSV) (Roost et al. 1995, Kalinke et al. 1996) (VSV in mice), tetanus as described by us in paper 1-3 and by others (Poulsen et al. 2007, de Kruif et al. 2009, Volk et al. 1984), and interestingly, HIV (Scheid et al. 2009) although this recent report contradicts the work by Köhler et al. (Kohler and Milstein 1992).

In several cases of pathogens, one specific antibody (or even Fab fragment) has been shown to be sufficient for clearance of the disease e.g. *P. aeruginosa* (Baer et al. 2009). In other cases, the binding of several antibodies is clearly necessary for neutralization, as has been shown for botulinum toxin neutralization (Marks 2004) and one could easily imagine this being true for other lethal toxins and complex or quickly mutating viruses. Hence, different antigenic targets require different numbers of antibodies for neutralization. Further, some viruses have been shown to require certain antibody isotypes for neutralization since complement activation is sometimes necessary (Benhnia et al. 2009).

The gene segment usage of specific antibody repertoires against several antigens has been shown to be highly biased just as the usage in naïve repertoires (Poulsen et al. 2007, Corbett et al. 1997, Souto-Carneiro et al. 2004, Yamada et al. 1991, Cox et al. 1994, Klein et al. 1993, Ohlin and Borrebaeck 1996). In paper 1 and 3, we report that our anti-tetanus repertoires exert a skewed gene segment usage. However, as we show in paper 1, they reflect natural skewing of non-specific repertoires. The skewing in both, V_H, J_H, V_κ and J_κ is quite similar for both donors and also between the different repertoires for the same donor. Interestingly, we found that our small primary repertoire actually displayed the same skewing as the mature responses. These findings indicate that

tetanus specific antibody repertoires do not converge against a certain type of rearrangement but keep reflecting the naïve repertoire bias and hence keep being diverse in terms of gene segment usage even after repeated exposure.

It is widely believed that the heavy chain CDR3 is the major contributor to the antigen-specificity for many antibodies (Xu and Davis 2000). Several groups have observed that some repertoire specificities require certain lengths of CDR3+FW4 for binding. These include antibody repertoires against dextran (19 residues), β-1,6 galactan (20 residues), and oxazolone (16 residues) (reviewed by (Berek and Milstein 1988)). However, as we show in paper 1, our anti-tetanus repertoires do not seem to require certain lengths of heavy chain CDR3 although there does seem to be a slight preference for lengths of 15 and 21 amino acids. Nevertheless, general skewing against longer heavy chain CDR3 loops has been observed in specific antibody repertoires (Ohlin and Borrebaeck 1996) such as west nile virus (Throsby et al. 2006), rabies virus (Kramer et al. 2005), tetanus toxoid (Poulsen et al. 2007) compared to unselected repertoires. Ohlin and Borrebaeck (Ohlin and Borrebaeck 1996) suggest that this might be due to potentially higher affinities for antibodies with longer heavy chain CDR3 loops because they allow for more contact points in the binding site than shorter CDR3s. However, Searle et al. have found that there is not a strict correlation between the number of contact points at the contact site and affinity of the antibody for the antigen (Searle 1995) so the significance of biases for longer CDR3s is not fully understood.

Based on phage display libraries with only one type of light chain but with heavy chains isolated from immunized donors, de Kruif et al. (de Kruif et al. 2009) argue that in tetanus repertoires, specificity is predominantly defined by the heavy chain CDR3s. Nevertheless, studies in other systems are contradictory. For example, Ohlin et al. (Ohlin and Borrebaeck 1996) have demonstrated that the reactivity of a high- affinity virus-neutralizing human antibody against the AD-2 epitope of cytomegalovirus gB can be modified by combining the original heavy chain with other light chains by phage display.

Some groups have speculated about the question of the absence of an equitable relationship between the number of antigen receptors and antigenic determinants (the paradox of infinite ligands for finite receptors) (Manivel et al. 2000, Joyce 1997, Mason 1998). It has been suggested that high flexibility of germline (primary) antibody paratopes in part compensate by cross-reacting with a large number of antigens (Manivel et al. 2000, Sethi et al. 2006, Brooks et al. 2008). This is in essence an amplification of the functional recognition repertoire beyond that prescribed by the BCR repertoire alone. The antibody paratopes are thought to be rendered more rigid by SHM in

conjunction with antigen-selection to ensure specific antigenic binding in conjunction with increased affinity (another role of somatic hypermutation) (Zimmermann et al. 2006). It has been discussed whether certain germline gene segments have undergone evolutionary selection for the generation of binding sites for certain dangerous pathogens that have frequently infected humans through evolution (Roost et al. 1995). Recently, Thomson et al. (Thomson et al. 2008) have argued that this is indeed the case for the antibodies expressed by repertoire dominating clones against HCMV (the segment being IGHV3). They observed that the contacts between the antigen and antibody did not change by the introduction of somatic hypermutations. Instead, the selected hypermutations stabilized and enhanced the germline encoded interaction with the antigen. They thus support the theories described above of germline antibodies being more flexible and hence more promiscuous than hypermutated antibodies. However, it does not seem highly likely that humans should be able to select for genes that counteract certain pathogens when almost all known pathogens mutate much faster than humans (our life cycle is much longer than most pathogens'). Further, with all the potential foreign antigens, it would be highly unlikely that one or several gene segments would specifically bind to certain targets although the suggested flexibility of germline antibodies could increase the number of targets. In paper 1 and 3 we report a broad usage of different V and J genes in all of the tetanus specific repertoires indicating that human antibody segments have not evolved to bind tetanus. Further, in paper 2 we report a constant addition of new antibodies to older repertoires and we find non-hypermutated antibodies of intermediate and high-affinity in paper 1 and 3. This suggests that the large potential of diversification of antibodies results in some antibodies having high affinity by chance rather than certain segments have evolved to specifically bind certain targets.

Peak antibody responses decline during the first year after vaccination, but antibody titers then stabilize and remain nearly constant for up to 75 years after immunization (Hammarlund et al. 2003, Crotty and Ahmed 2004, Slifka 2004, Crotty et al. 2003). Höfer et al. (Hofer et al. 2006) have calculated that tetanus as a model antigen produces a number of antibody producing tetanus-specific plasma cells which drops to 50% 173 years after immunization. Indeed, a recent study by Yu et al. (Yu et al. 2008) on survivors of the 1918 H1N1 influenza virus pandemic confirms the prolonged presence of specific antibodies. The study demonstrates that even nearly 90 years after exposure to this particularly virulent virus, 7 out of 8 tested blood samples from survivors still contained B cells secreting antibodies that bound the 1918 hemagglutinin (HA). Strikingly, all 32 individuals tested

showed seroreactivity with the 1918 virus strain. Out of five isolated monoclonal antibodies from these individuals, none cross-reacted with more contemporary human influenza viruses. This demonstrates that for some antigenic targets (and maybe particularly viruses), circulating memory B cells can be found for nearly a hundred years – or rather throughout the person's lifetime. Similar findings have been reported by Crotty et al. (Crotty et al. 2003) who find that Vaccinia-specific serum titers are kept constant up to at least 60 years after immunization.

In paper 1, we demonstrate that the specific kappa antibody repertoire against tetanus consists of in the order of 100 clonotypes and hypermutated variants thereof based on the first antibody repertoire from each of the two hyperimmune donors. The finding is confirmed in paper 2, where the two consecutive repertoires from each donor verify the primary estimates on repertoire sizes. Further, we demonstrate in paper 2 that the influx of new clonotypes into existing tetanus repertoires upon a new challenge is relatively large and may compose over half of the "base" repertoire. This suggests that the composition of specific antibody repertoires is relatively dynamic while repertoire sizes (at least against tetanus) seem to remain about the same. The size of the total B cell repertoire only allows the expression of a fraction of the genetic repertoire at any given time, thus only a random sample of the total potential is available (Berek and Milstein 1988). It therefore seems likely that only relatively small numbers of clonotypes are specific for each target and the specific numbers could be even smaller in the case of structurally restricted antigens such as e.g. Rhesus D (Andersen et al. 2007).

6. Affinity maturation

The concept of affinity maturation was introduced by Siskind and Benacerraf (Siskind and Benacerraf 1969). The development of the concept followed the 1965 demonstration by Siskind and Eisen that there was a gradual increase in affinity of specific serum antibodies after small amounts of antigen were repeatedly injected in rabbits (Siskind and Eisen 1965). Parts of the affinity maturation process still need to be uncovered. However, there is a general agreement that affinity maturation of B cells is initiated by encounter with antigen which in turn triggers initiation of somatic hypermutation of the Ig genes. B cells are then competitively selected for high affinity to specific antigen by the signaling to certain clones to proliferate faster as they acquire advantageous mutations compared to other clones as explained by the clonal selection theory (Burnet 1959). Clones whose hypermutations result in either non-functional or self-binding antibodies are deleted during this process or undergo further rearrangement processes (e.g. receptor editing). Therefore, maturation is the result of selection and specific expansion of B cell clones expressing high-affinity antibodies (Berek and Milstein 1987). Later it has been demonstrated, that the process of affinity maturation takes place in the germinal centers of secondary lymphoid tissues and is indeed characterized by somatic hypermutation of Ig genes and subsequent selection of the clones with genes encoding the best antibodies (MacLennan 1994).

The end-goal of the affinity maturation process is to maximize (or in praxis, optimize) for complementarity between epitope and paratope (Manivel et al. 2000). Nevertheless, an affinity threshold has been suggested for B cell triggering (Sale et al. 2001, Batista and Neuberger 1998). It has been argued that if the threshold is set too high, the B cell repertoire might not cover widely enough, while a too low threshold might be associated with excessive B cell activation and difficulties in discrimination between self and non-self (Batista and Neuberger 1998). Sale et al. have even speculated that there might be a difference in this triggering threshold during the development of B cells (immature B cells) and for mature B cells.

In 1995, Foote & Eisen proposed that *in vivo* selection of antibodies must have an affinity ceiling (Foote and Eisen 1995). This proposal was based on assumptions on association and dissociation rates. Maximum selection on on-rates (k_{on}) was predicted to be 10^5-$10^6 M^{-1}s^{-1}$ based on diffusion coefficients of antigens which had been verified experimentally (Northrup and Erickson 1992, Raman et al. 1992). Limiting dissociation rates (k_{off}) of 10^{-3}-$10^{-4}s^{-1}$ were estimated by multiplying the rate of receptor internalization upon antigen binding by a factor 2-3 (Foote and Eisen 1995)

where half-life for internalization was estimated to 8.5 minutes based on endocytosis (Watts and Davidson 1988). Thus, the predicted maximum k_{on} values of 10^5-$10^6 M^{-1}s^{-1}$ and lower k_{off} limits of 10^{-3}-$10^{-4}s^{-1}$ lead to an affinity ceiling having equilibrium dissociation constants (K_D) in the range of 10^{-8}-10^{-10} M. This does not mean that antibodies of higher affinity do not arise as both we (Poulsen et al. 2007) and others have demonstrated (Roost et al. 1995, Wrammert et al. 2008, Rathanaswami et al. 2005). Rather antibodies with affinities above the ceiling have no further advantage in antigen selection during normal responses. Although, Batista and Neuberger (Batista and Neuberger 1998) have demonstrated *in vitro* that these theoretical boundaries seem to exist, the boundaries have been difficult to address experimentally *in vivo*. This has mainly been due to the inability of existing antibody cloning techniques to generate a sufficient number of antibody clones from single antigen repertoires of animal or human origin. Recent advance in techniques for isolation and analysis of single antibody producing B cells has lifted this limitation and thereby allowed for detailed studies of the composition and functionality of antigen-driven antibody repertoires at single time points (Poulsen et al. 2007, Love et al. 2006, Yu et al. 2008, Lanzavecchia et al. 2007, Wrammert et al. 2008, Scheid et al. 2009). In paper 1, we show that affinities of *in vivo* selected antibodies cover a span of a million fold. We further demonstrate that both affinities and binding kinetics (on- and off-rates) follow log-normal distributions reflecting the intrinsic properties of random somatic diversification. In paper 2, we report for the very first time, that affinities for *in vivo* selected antibodies binding to tetanus toxoid peak at $1x10^{-9}$ M at physiological temperature (37°C). Further, we are able to demonstrate that kinetic rate constants are maturated separately with constants peaking at $1.6x10^5$ $M^{-1}s^{-1}$ and $1.7x10^{-4}$ s^{-1} at physiological temperature for on- and off-rates, respectively. The observed limits are in agreement with the theoretical limits proposed by Foote and Eisen (Foote and Eisen 1995).

Because relatively short time passed between the second and third vaccinations in our study, there is a slight possibility that the observed ceiling is a bit lower (higher K_D) than what is actually possible due to much available antigen. If antigen persists in the system for a longer period of time it does not allow for maximum competition which leads to a case where the very high affinity clones do not have the extreme selective advantage they would have at low amounts of available antigen. However, we do not expect this feature to be able to displace the ceiling by several orders of magnitude.

In paper 2 we reported that on-rates peak before off-rates since they did not improve with several challenges whereas the off-rates were significantly slower (and hence better) in the second and third

repertoires compared to the first. However, the off-rates may be under a more tight control since they are less dispersed than on-rates. The observed separate maturation of kinetic rate constants supports that maturation of antibody responses is kinetically rather than thermodynamically controlled (Foote and Milstein 1991, Furukawa et al. 1999, Roost et al. 1995).

The antibody affinity ceiling we observe is not only consistent to theoretical limits under certain assumptions. Batista and Neuberger (Batista and Neuberger 2000) have shown *in vitro* that the amount of antigen needed to trigger a B cell response is discriminated by the cell over a range in affinities of 10^{-6}-10^{-10} M. With greater affinities (lower K_D), there was no discrimination for B cells recognizing soluble antigen. However, they found that the form in which the antigen was displayed to the B cell affected the ceiling. They demonstrated that particulate antigen or antigen tightly tethered to a non-internalizable surface displayed lower affinity ceiling values (higher K_D) – as low as 10^{-6} M – possibly driven by higher avidity. Our findings in paper 2, that the affinity ceiling of 10^{-9} M for *in vivo* selected tetanus specific antibodies suggest that most of the tetanus toxoid is displayed in a soluble form or that other mechanisms have influence on *in vivo* selection of antibodies.

As described, we have observed that affinities cover a span of a million-fold which was confirmed by data presented in paper 2. This large span can be explained by the observation that hypermutation occurs locally in distinct germinal centers, allowing for local competition of B cell clones but that there is no competition between distinct germinal centers (Dal Porto et al. 1998, Jacob et al. 1991, Jacob and Kelsoe 1992, Kelsoe and Zheng 1993). Further, Jacob & Kelsoe demonstrate that both focus and germinal center populations are pauciclonal, founded, on average, by three or fewer B lymphocytes (Jacob and Kelsoe 1992). These very small numbers of cells forming the basis of a GC offer a nice explanation for a large span in affinities as a result of limited local competition. Further, the explanation is supported by our observation in paper 2 that a relatively large part of the anti-tetanus repertoire consists of new clones entering the repertoire – these new clones may even compose over half of the "base" repertoire. Naturally, these clones contribute to the large span in affinities but as we have shown in paper 1 even newly formed antibodies can be of high affinity. Therefore, they might even contribute to affinities in the full span and not only in the lower end.

The large span in affinities and the large influx of new clonotypes into existing tetanus repertoires upon a new challenge combined with the finding that the primary repertoire is relatively diverse and can include high-affinity binders, suggest that each subsequent repertoire consists of a memory

repertoire and a primary repertoire. In other words, it seems that a part of the mature repertoire consists of specific antibodies from newly activated and differentiated naïve B cells. The pauciclonality of GCs may easily make room for expansion of these newcomers. The theory is supported by the fact that the span in affinities is approximately the same for antibody repertoires that seem to have reached an affinity ceiling as repertoires that have not (data in paper 2). Further, the fact that we are able to isolate a number of tetanus specific non-hypermutated antibodies in mature antibody repertoires is in favor of this theory as well.

7. Concluding remarks

In this thesis, different aspects of antibody repertoires are addressed with the overall goal to increase the understanding of genetic and functional development of *in vivo* selected specific human antibody repertoires. We anticipate that these results concentrating on antibody repertoires against tetanus toxoid can be transferred to a more general understanding.

In paper 1, we characterized the composition of antigen-specific polyclonal antibody responses. Using the Symplex technology, we isolated antibody repertoires from two healthy donors after receiving a tetanus vaccination. We argued that the observed genetic diversity among the tetanus toxoid specific plasma cells indicated that human polyclonal repertoires are limited to the order of 100 B cell clones and hypermutated variants thereof. Further, we found that affinity and kinetic binding constants were log-normally distributed and that antibody affinities varied a million-fold. We also identified antibodies of high affinity without hypermutations indicating that antibodies selected from the naive repertoire are not only of low affinity.
In paper 2, we isolated further two consecutive tetanus specific repertoires from each of the two donors from paper 1. We demonstrated that maturation of on- and off-rates occurred independently which indicated a kinetically controlled affinity maturation process. The repertoires developed functionally from the first to the second vaccination while the third vaccination of each donor induced no significant changes in the distribution of somatic mutations and binding rate constants. This implied that the limits for affinity maturation and repertoire diversification had been reached. The fully matured antibody repertoires remained similar in size to earlier isolated repertoires and also remained genetically diverse and dynamic. Somatic mutations showed normal distribution profiles while kinetic rate constants confirmed the log-normal distributions demonstrated in paper 1. The fully matured antibody repertoires allowed us to report for the first time an experimental determination of the biological limits for antigen-driven affinity maturation and repertoire diversification. The clear distribution profiles of somatic hypermutations, on- and off-rates, and affinities indicated that mean values could be considered as biological constants defining the observed boundaries. The mean level of somatic mutations was 14 and 7 amino acid transitions per V_H and V_κ gene segment, respectively. At physiological temperature, affinity maturation peaked at on-rates of $1.6x10^4 M^{-1}s^{-1}$ and off-rates of $1.7x10^{-4}s^{-1}$ leading to maximum mean affinity of $1.0x10^{-9}M$.

In paper 3, we investigate the role of diversification mechanisms in developing antibody repertoires. In addition to the hyperimmune repertoires from paper 1 and 2, we isolated an antibody repertoire from a tetanus naïve donor after receiving the first tetanus vaccination. We demonstrated that the diversity of tetanus specific antibody repertoires was evident after a single vaccination and did not change with many antigenic challenges. Also, we demonstrated that as early as day 6 after the first vaccination, the level of mutations of each specific antibody was surprisingly high with an average of 15 amino acid mutations per antibody. In our large data sets, we did not observe any instances of receptor editing suggesting that this is not an important mechanism for antibody diversification. Further, we identified few antibodies with deletions and none with insertions – indicative of a minor contribution by these processes to antibody diversification.

The number of hypermutations for each antibody did not correlate to affinity or on- rates confirming our previous results. However, off-rates were improved when mutations were introduced to germline antibodies. Based on these observations we concluded that somatic hypermutations are important for improvements in off-rates of germline antibodies. This was supported by our finding that somatic hypermutations occur very fast in the primary tetanus repertoire. Our findings indicated that the quality of the primary antibody repertoire against physiologically relevant antigens is better than predicted from hapten studies.

Together, the results of this project have increased the understanding of compositions and development of specific human antibody repertoires following immunization. Therefore, the results may be of use in the development of future antibody therapeutics and in vaccine design. Further, the findings are of general immunological interest since the conclusions regarding repertoire size and functional maturation limits of tetanus specific repertoires may well be applicable to antibody repertoires of other specificities.

7.1 Suggestions for further work

The results of this project have opened up for many further questions that could be sought answered by treatment of the existing or new Symplex antibody repertoires:

1. Does the specific repertoire cover the antigen or are there certain dominant epitopes?

2. How do human antibody repertoires change on the molecular level with multiple exposures to the same antigen – and are there differences between individuals?

3. Is the diversity of the mouse antibody repertoire comparable to that of humans on genetic and functional levels?

4. What is the starting point for selection of antibodies from a naïve repertoire?

5. What is the size of the naïve antibody repertoire at any given time in humans?

Question 1 could be sought answered simply by using the previously expressed and purified human Fab fragments to test how well the repertoire covers the antigen by epitope mapping. The experiments would be conducted on SPR equipment and would reveal if the functional diversity of a repertoire works by full coverage of the antigen or whether it only covers a few immuno-dominant epitopes. In addition, it would reveal how many antibodies from a given repertoire that can bind to the antigen simultaneously. The results of these studies would be of fundamental immunologic interest as well as supportive to future vaccine design.
Regarding question 2, a further genetic analysis of the seven existing antibody repertoires from the two hyperimmune donors could aid in characterizing the development of antibody repertoires during several immunizations with a single antigen. This study could employ bioinformatics to analyze the genetic details of developing antibody repertoires and to characterize selection mechanisms shaping human antibody repertoires. The data could further be analyzed to show whether there is a difference between individuals. The results would help reveal rules guarding the generation and expansion of antibody diversity in human B cells and the optimization of antibody structures that bind microbial antigens. This knowledge would help to better understand the

influence of affinity maturation in the maintenance, expansion, or narrowing of antigen-specific repertoires in the B cell compartment. It could be very supportive in the future design of vaccines. A further investigation of human antibody responses would also assist the development of recombinant mono- or polyclonal antibody-based treatments since it would aid in the selection of antibodies for more potent and efficient treatments.

Since the Symplex technology has already been employed in the isolation of anti-tetanus mouse repertoires, these could be used in the answering of question 3. By analyzing the genetic and functional differences between human and mouse repertoires, important knowledge on the comparability between human and mouse antibody repertoires could be obtained. The results could be of importance to the interpretation of the many studies within immunology that are conducted in mice for practical and ethical reasons. Hence, the study would help elucidate how transferable mouse study results are to humans.

To answer question 4 and investigate where humoral immunity begins in humans could be done by characterizing more antibody repertoires upon the first tetanus vaccination in donors and antibody repertoires upon the second tetanus vaccination of these donors. The study could also include the characterization of a panel of tetanus specific antibodies in germline configuration. This study would reveal important information about initial selection mechanisms of antibodies. This could be done by choosing a panel of ~25 antibodies, which we have previously characterized functionally, and remove all hypermutations by gene synthesis. The antibodies should be functionally characterized (affinities) and compared them to their original antibody counterparts. This would further address the issues of whether antibodies start out as low affinity antibodies that only get better affinities by the process of affinity maturation since results from our previous studies on a few non-mutated antibodies show that some antibodies actually have a high affinity before somatic hypermutations are introduced (see papers 1-3). The generation of a non-hypermutated version of an antibody repertoire selected *in vivo* would be an important and unique opportunity to study a snapshot of the same antibody repertoire before and after somatic hypermutation and affinity maturation. Further, it could be valuable in investigating further whether certain antibody gene segments have been developed through evolution to specifically target tetanus. Therefore this study could give insights into the basis on which antibody-producing cells are chosen for expansion and differentiation in the initial phase.

Question 5 could be sought answered by the development of mathematical and statistical methods for predictions on the size of the naïve antibody repertoire based on the specific repertoires

characterized. Thus, these studies could reveal important fundamental knowledge about selection mechanisms that take effect upon the first encounter with antigen as well as the pool of cells that are the basis for selection. This knowledge could help determine whether people who have been exposed to dangerous microbial agents only once (and which we cannot vaccinate against) can be used for isolation of antibody repertoires that can form the basis for the development of a successful drug against the harmful agent in question.

It would be of great value to be able to compare these repertoires: the “non-hypermutated”, the initial and second natural repertoires and analyze them statistically. Additionally, it would be valuable to compare the affinities of the naturally hypermutated repertoires from the multichallenged and hence hyperimmune donors.

The studies presented above could generate information which would be valuable for the development of new drugs such as vaccines and antibody therapeutics - both by a better prediction of B cell epitopes and by a better understanding of the development of protecting antibody repertoires. Additionally, the results would contribute to the field of fundamental immunology by a better understanding of B cell epitopes and by elucidation of principles guarding antibody selection and development of antibody repertoires. The results of these suggested studies may therefore be of both fundamental immunological interest and of industrial and societal relevance.

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Paper 1

Kinetic, Affinity, and Diversity Limits of Human Polyclonal Antibody Responses against Tetanus Toxoid

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Due to technical limitations, little knowledge exists on the composition of Ag-specific polyclonal Ab responses. Hence, we here present a molecular analysis of two representative human Ab repertoires isolated by using a novel single-cell cloning approach. The observed genetic diversity among tetanus toxoid-specific plasma cells indicate that human polyclonal repertoires are limited to the order of 100 B cell clones and hypermutated variants thereof. Affinity and kinetic binding constants are log-normally distributed, and median values are close to the proposed affinity ceilings for positive selection. Abs varied a million-fold in affinity but were restricted in their off-rates with an upper limit of 2×10^{-3} s^{-1}. Identification of Abs of high affinity without hypermutations in combination with a modest effect of hypermutations on observed affinity increases indicate that Abs selected from the naive repertoire are not only of low affinity but cover a relatively large span in affinity, reaching into the subnanomolar range. *The Journal of Immunology,* **2007, 179: 3841–3850.**

Antibodies are produced by B cells in response to antigenic challenge. The primary repertoire of B cells is made by a random rearrangement of five gene segments, including *V*, *D*, and *J* gene segments for the H chain and V and J gene segments for the L chain. In addition, junctional diversity is introduced by nontemplate insertion and deletion of nucleotides in the joining regions during the rearrangement process. B cell clones from the primary repertoire are selected by Ag and subjected to a clonal expansion/selection process in which hypermutation occurs, thereby increasing the affinity of the Ab/Ag interactions (1). Consequently, the humoral immune response consists of a polyclonal repertoire of B cells generally characterized by a diverse genetic profile and the production of high affinity Abs. However, the understanding regarding the development and composition of polyclonal Ab repertoires in response to Ag challenge is modest, mainly due to the limitations of current techniques.

The functional development of Ag-selected polyclonal Ab repertoires has mainly been studied by making mAbs from mice progressively exposed to Ag. By studying anti-hapten responses it has been concluded that primary responses are of low affinity, which increases as a consequence of somatic mutations upon multiple challenges with Ag (2–4). However, anti-hapten responses are known to be genetically restricted and involve only a limited set of V gene segments (discussed below), implying that the Ag has a significant role in shaping such Ab repertoires. In contrast, high-affinity Abs have been observed early in mice in response to a viral infection (5) and to hen egg lysozyme (6), implying that primary Ab responses toward more complex Ags are less restricted in terms of affinity. Foote and Eisen (7) proposed a theoretical limit for Ab affinities in developing B cell responses based on the consideration that the selection based on the off-rate must be limited by the rate of Ag endocytosis, leading to maximal off-rates in the range of 10^{-3} to 10^{-4} s^{-1} and that the on-rates cannot exceed the maximal rate of diffusion in the range of 10^5 to 10^6 M^{-1} s^{-1}. Thus, the immune system should not be able to select for affinity constants (K_D) beyond 0.1 nM, although such Abs might arise by chance.

Genetic diversity of Ag-selected polyclonal Ab repertoires toward complex Ags has mainly been studied by using phage display technology because of its ability to screen massive numbers of V gene sequences. As a recent example, ~55 uniquely rearranged H chain variable domain (V_H)[2] sequences were isolated by phage display from two human donors immunized with a rabies vaccine (8). However, phage display involves the random pairing of H and L chain genes, which often leads to a drop in affinity. Consequently, only about one-third of the anti-rabies Abs appear to be of high affinity. In addition, phage display preferentially selects for promiscuous V_H domains, which further reduces the diversity of the Ag-specific repertoires isolated by phage display (9, 10). Thus, although it is currently the preferred method for studying the composition of Ag-specific repertoires, phage display is likely to underestimate the actual V_H diversity generated by the immune system and, further, provides no information regarding cognate L chain usage and, hence, native affinities.

We have recently reported a novel strategy, the Symplex Technology, for the isolation of comprehensive repertoires of cognate pairs of Ab V genes directly from human plasma cells (10). The screening of such V gene repertoires allows for simultaneous analysis of the clonal and functional diversity of the Ag-selected human Ab repertoire. In this study we examine the genetic diversity and binding characteristics of two comprehensive panels of distinct mAbs obtained from single individuals immunized with a tetanus toxoid (TT) vaccine. Our results have implications for the understanding of the mechanisms that lead to a polyclonal response and for the limits of the selective forces responsible for affinity maturation.

Materials and Methods

Donors and vaccination

Two healthy volunteers were boosted with a TT vaccine (Statens Serum Institut, Copenhagen, Denmark) in the musculus deltoideus. Both donors

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[2] Abbreviations used in this paper: V_H, H chain variable domain; J_H, H chain joining region; SPR, surface plasmon resonance; TT, tetanus toxoid; V_κ, κ-chain variable domain; J_κ, κ-chain joining region.

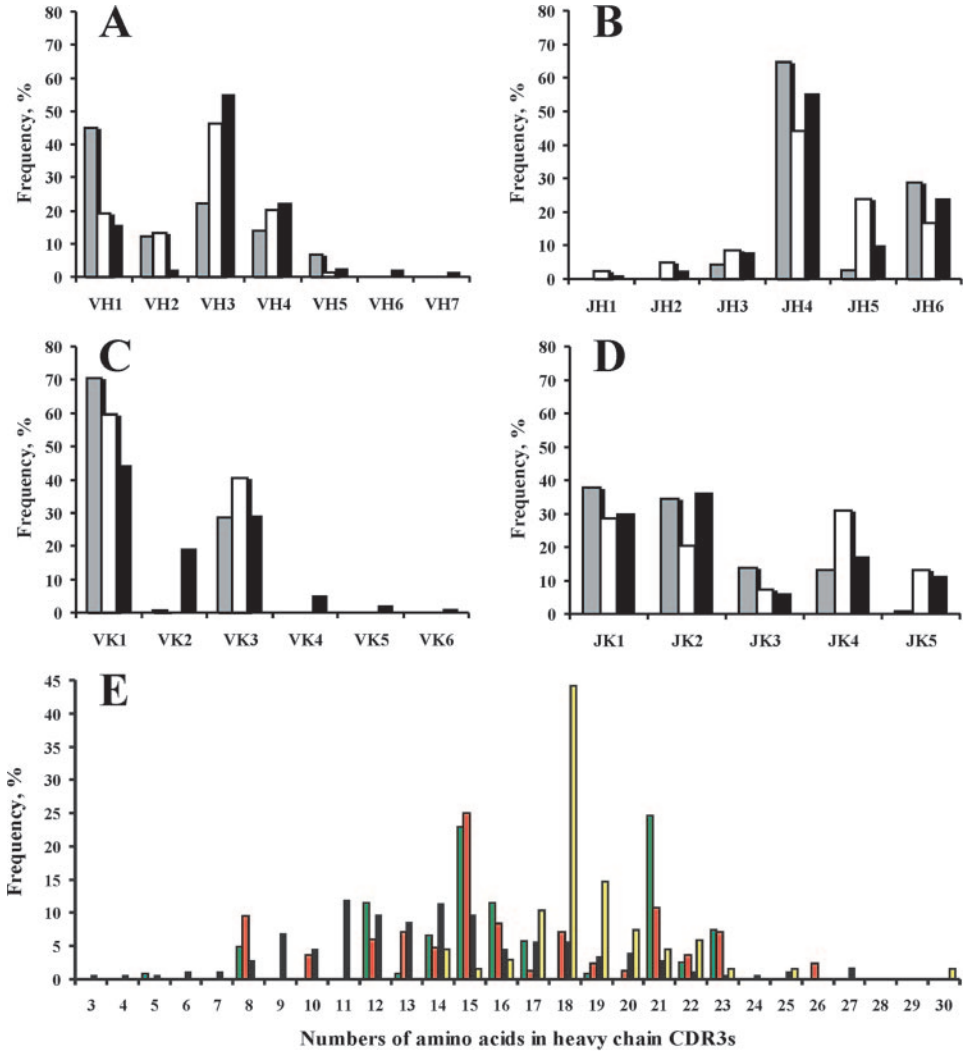

FIGURE 1. *V* and *J* gene segment usage and distributions of H chain CDR3 lengths. Distribution of H chain gene segment usage, V_H (*A*) and J_H (*B*), and L chain gene segment usage, V_κ (C) *and* J_κ (*D*) in the Ab repertoires of TT01 (gray bars; $n = 122$), TT02 (open bars; $n = 84$), and a previously described naive peripheral IgM^+ B cell repertoire (13, 14) (filled bars, $n_{V_H} = 350$, $n_{J_H} = 206$, $n_{V_K J_K} = 321$). H chain CDR3 lengths (*E*) of the present set of sequences from donor TT01 (green bars; $n = 122$) and TT02 (red bars; $n = 84$) in comparison to a set of randomly selected H chain CDR3 lengths (black bars; $n = 177$) (18) as well as a set of CDR3 lengths of highly restricted anti-rhesus D Abs (yellow bars, $n = 68$) (15).

were Caucasian adults (designated TT01, a male, and TT02, a female) residing in Denmark and both had been challenged with TT several times before this study. Six days after the vaccination, blood (200 ml) was collected in anticoagulant. The project was approved by the Regional Ethical Review Board in Copenhagen, Denmark, and informed consent was obtained from each donor.

Ab libraries were constructed according to Meijer et al. (10) from plasma blasts isolated on a FACSAria cell-sorting system (BD Biosciences) from freshly drawn blood. Shortly, PBMC were isolated using Lymphoprep (Axis-Shield) and enriched for B cells by a $CD19^+$ MACS procedure. B cells that stained positive for CD19, highly positive for CD38, and intermediately positive for CD45 were single cell-sorted directly into wells of 96-well PCR plates and designated plasma cells. H chain and κ L chain variable region genes were linked and amplified by RT-PCR. Amplified genes were pooled and inserted into a bacterial Fab expression vector and transformed into *Escherichia coli*. Fabs were expressed and screened for activity against TT by ELISA. All positive clones were sequenced and the V_H-V_κ sequences were aligned to group clones according to sequence homology. For each group, the V-D-J usage and location of somatic mutations were determined by alignment with germline sequences using the ImMunoGeneTics sequence directory (www.ebi.ac.uk; http://imgt.cines.fr). The phylogenetic trees were generated using ClustalW (www.ebi.ac.uk).

Construction of deduced germline Fabs

Protein sequences of two Fabs without hypermutations were constructed by aligning mutated Ab sequences from common rearrangements in the two donor repertoires (V_H1–69-J_H6 $V_\kappa1$–27-$J_\kappa3$ and V_H3–21-J_H6 $V_\kappa1$–39-$J_\kappa1$; where J_H is H chain joining region) to germline protein sequences of *V* and *J* segments (http://imgt.cines.fr). The more common CDR3 sequence motifs of each H and L chain rearrangement were inserted in each synthetic protein sequence (underlined in Figs. 2 and 3). Each protein sequence was back translated and *Asc*I and *Xho*I restriction sites were added to the ends of V_H sequences. Furthermore, an L chain κ constant domain was added to each L chain variable region and *Nhe*I and *Not*I restriction sites were added to the ends of L chain genes. Finally, the genes were optimized for expression in *E. coli* and synthesized (GeneArt). Each synthesized gene was cloned into a vector and plasmid DNA was purified from transformed *E. coli* (GeneArt). Each gene was released from its vector with the appropriate combination of restriction enzymes, and fragments were subsequently purified by preparative 1% (w/v) agarose gel electrophoresis. L chain genes were subcloned into pJSK301. Subsequently, V_H regions were subcloned into the L chain gene-containing vectors. Plasmid DNA was sequenced (AGOWA) and verified. Expression of TT-specific Fab expression was verified by ELISA.

Multiple alignments

A genetic phylogeny analysis was conducted to assess the homology of all 206 Abs from donors TT01 and TT02. A phylogenetic cladogram (data not shown) was generated by submitting FASTA formats of variable region amino acid sequences (combined H and L chain sequences) of all Abs to the European Molecular Biology Laboratory-European Bioinformatics Institute online phylogeny analysis program, ClustalW (http://www.ebi.ac.uk/clustalw/) (11), using default settings. The cladogram containing only affinity-measured Abs (see Fig. 5) was generated similarly.

Statistical calculations of repertoire sizes

According to Behlke et al. (12) the probability of observing exactly *d* distinct gene rearrangements among a total number of *r* examined sequences for a fixed number *n* of distinct gene rearrangements is shown in Equation 1,

Cluster 1

Heavy chain	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH1-69 JH6	SGAEVKKPGSSVKVSCKAS	GGTFSSYA	ISWVRQAPGQGLEWMGG	IIPIFGTA	NYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYC		WGQGTTVTVS
1-60D04	.....	..S..T..	.T.....	.....ASR	D.....V...E..R.V.....D.....	.....	.....M....
1-60H10	.....	..S..T..	.T.....	.....ASR	D.....E..R.V.....D.....	.....	.....
1-62D09	.....	..S..T..	.T.....	.....ASR	D.....E..R.V.....D.....	.....	.....L....
1-60H02	.....	..S..T..	.T.....	.....ASR	D.....E..R.V...PR.....	.....	.....L....
1-62A10	.....	..S..T..	.T.....	.....ASR	D.....E..R.V....R.....	.....	.....
1-62H03	......R.....	..S..T..	.T.....	.....ASR	D.....E..R.V....R.....	.....	.....M....
1-63A02	.....	..S..T..	.T.....	.....ASR	D.....E.SR.V....R.....	.....	.....
1-62D07	.....	..S..T..	.T....V.....	.....ASR	DC.....E..R.V.....	.....	.....L....
1-63B12	.....	..S..T..	.T....V.....	.....ASR	D.....E..R.V.....	.....	.....
1-60D05	.....T.	..S..T.S	.T.....	.N...A.R	D.....E..R.V....RN.....	...F.....	.....L....
1-61A04	.....T.	..S..T.S	.T.....X.....	.N...A.R	D.....E..R.V....RN.G.....	...F.....	.....
1-61H12	.....NN.....T.	..S..T.S	.T.....	.N...A.R	D.....E..R.V....RN.....	...F.....	.....L..--
1-62D12	.....T.	..S..T.S	.T.....	.NL..A.R	D.....E..R.V....RN.....	...F.....	.....
1-62E04	.....T.	..S..T.S	.T.....W.....	.N...A.R	D.....E..R.V....RN.....	...F.....	.....
1-60G11	.....	..S..T.I	FT.....	.N...A.R	D.PK.....E..R.V.....T.....	.....	.....
1-60F09	.....TE	.....T.V	...M.....	.V...N.P	.....R.....R..G.....	...V....S....GF	.....
1-63C07	.....	.....T.S	.....	.T....AP	K.G.....GI.....N.V...M...K.D.....	..DY....H....DA	.....M....
1-63D07	...A.TT.....T....V.	.A...T..	VN.....	.T....PT	-....Y...AK.....V..Q.TR.T.....IIF.	..DR....HH.YMD.	..K.....
1-62H07	...A.TT.....T....V.	.A...T..	VN.....	.T....PT	-....Y....K.....V..Q.TR.T.....IIF.	..DR....HH.YMD.	..K.....--
Cluster CDR3 consensus						ARVLGGTRLYYALNV	

VH3-21 JH6	SGGGLVKPGGSLRLSCAAS	GFTFSSYS	MNWVRQAPGKGLEWVSS	ISSSSSYI	YYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYC		WGQGTTVTVS
1-60B12	.....	..S..NNN	.....T.....A.	..FG.H..	S.....R.AV.....V.....	T.CR....T..YMD.	..K..L....
1-63B09	.....	..S..NNN	.....T.....A.	..FG.H..	S.....R.AV.....V.....	T.CR....T..YMDI	..K..L....
1-60C03	.....	.SS..NNN	.....T.....A.	..FG.H..	S.....R.AV.....V.....	T.CR....T..YMD.	..K..L....
1-60F06	.....	..S..NNN	.....T.....A.	..FG.H..	S.....R.AV.....V.....	T.CR....T..YMD.	..K..L....
1-60D08	.....	..S..NNN	.....T.....A.	..FG.H..	S.....R.AV.....V.....	T.CR....T..YMD.	..K..M....
2-135A03	.....	....RNKV	.....	.TGTGD..	D.G.....V.....N.L.F....N..P.....F.	..Y..S..G..YMD.	..K..M....
2-135B11	.....	....RNKV	.....	.TGTGD..	D.G.....SV.....N.L.F....N..P.....F.	..Y..S..G..YMD.	..K..M....
Cluster CDR3 consensus						ARVLGGTRLYYALNV	

Light chain	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VK1-27 JK3	SPSSLSASVGDRVTITCRAS	QGISNY	LAWYQQKPGKVPKLLIY	AAS	TLQSGVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	FT	FGPGTKVDIK
1-60D04	F.....	.....F	.....E....	G..	.....AE.....N.....	.....R..	.....
1-60H10	.....	.....F	.....E....	G..	.....E.....N.....	.....R..	.....
1-62D09	.....	.....F	....H.....E....	G..	.....E.....N.....	.....R..	.....
1-60H02	.....	.....F	.....R..E....	G..	.....N.....	.....R..	....A.....
1-62A10	.....	.....F	.....E....	G..	.....N.....	.....R..	.....
1-62H03	.....	.....F	.....E....	G..	.....N.....	.....R..	.....R
1-63A02	.....	.....F	.....E....	G..	.....N.....	....G..R..	.....
1-62D07	.....	.....F	.....N....	G..	.....T.....	.....R..	.....
1-63B12	.....	.....F	.....N....	G..	.....T.....	.....R..	.....
1-60D05	.....	.....F	...H.....Q.....	G..	.....NG.....	.....	.....R.E..
1-61A04	...A.....	.....F	...H.....Q.....	G..	.....NG.....C.	.....	.....R.E..
1-61H12	.....	.....F	...H.....Q.....	G..	.....NG.....	.....	.....R.E..
1-62D12	.....	.....F	...H.....Q.....	G..	.....NG.....	.....	.....R.E..
1-62E04	.....	.....F	...H.....Q.....	G..	.....NG.....	.....	.....R.E..
1-60G11	.....S....	.....F	...F.....Q.....	V..	.....S...NG.....	.R.....	.....E..
1-60F09	.....S.....	.....F	.....K.....R....	G..	.....	.E.SNA....	.....H..
Cluster CDR3 consensus						QKYDSGLI	

VK1-27 JK1	SPSSLSASVGDRVTITCRAS	QGISNY	LAWYQQKPGKVPKLLIY	AAS	TLQSGVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	WT	FGQGTKVEIK
1-63C07	.....	.....F	.....R..R....	S..	.....	...N.DP-R.	.....
Cluster CDR3 consensus						QKYDSGLI	

VK3-20 JK2	SPGTLSLSPGERATLSCRAS	QSVSSSY	LAWYQQKPGQAPRLLIY	GAS	SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	YT	FGQGTKLEIK
1-63D07	.....	..F.GDF	.....R.....	...	T.....TS.....	.Q.-..PLR.	.....
1-62H07	.....	..F.GDF	.....R.....	...	T.....TS.....	.Q.-..PLR.	..R.....
Cluster CDR3 consensus						QKYDSGLI	

VK3-20 JK1	SPGTLSLSPGERATLSCRAS	QSVSSSY	LAWYQQKPGQAPRLLIY	GAS	SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	WT	FGQGTKVEIK
1-60B12	.....A....D.....G..	...FGDF	.....H.....	...	T.....A.....F.	.*.G..--VF.	.....LG..
1-63B09	.....A....D.....G..	...FGDF	.....H.....	...	T.....A.....F.	.R.G..--VF.	.....L...
1-60C03	.....A....D.....G..	...FGDF	.....H.....	...	T.....A.....F.	.Q.G..--VF.	.....L...
1-60F06	.....A....D.....G..	...FGDF	.....H.....P...	...	T.....A.....F.	.Q.G..--VF.	.....L...
1-60D08	.....A....D.....G..	...FGDF	.....H.....	V..	T.....A.....F.	.Q.G..--VF.	.....L..R
Cluster CDR3 consensus						QKY-DSGLI	

VK3-20 JK3	SPGTLSLSPGERATLSCRAS	QSVSSSY	LAWYQQKPGQAPRLLIY	GAS	SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	FT	FGPGTKVDIK
2-135A03	...I.....	...INNF	.....	...	.....G.....	.Q.G.SPPI.	.....
2-135B11	...I.....	...INNF	.....	...	.....G.....	.Q.G.SPPI.	.....
Cluster CDR3 consensus						QKYDSGLI	

FIGURE 2. Alignment of cluster 1 V_H and V_κ gene amino acid sequences. The CDR3 consensus motif of each H and L chain cluster is indicated below each group of sequences. Dots represent amino acids similar to the germline sequence or the CDR3 consensus motif. Amino acids that differ from those found at the same position in the germline/consensus sequence are indicated by the letter of the replaced amino acid. A deletion is indicated by a dash (-). Each clone is denoted by the donor number (1 or 2, corresponding to donor TT01 and TT02, respectively) followed by the clone name (e.g., 60D04).

$$P(d) = S(r,d)\binom{n}{d}d!n^{-r} \quad (1)$$

where $S(r,d)$ are Stirling's numbers of the second kind. The analysis assumes that no significant skewing of the data set by nonrandom gene segment usage has occurred.

For donor TT01 the maximum likelihood estimate of n was $n = 29$ for d and r fixed at d = 29 and r = 122, whereas the maximum likelihood estimate for donor TT02 was $n = 48$ for $d = 40$ and $r = 84$. A 95% one-sided confidence of the estimates were calculated by determining the smallest value of n for which the probability of observing d distinct gene rearrangements among r examined sequences was $<5\%$. This returned a value of $n = 31$ for donor TT01, whereas the 95% confidence for donor TT02 was bound at $n = 57$.

Surface plasmon resonance (SPR)

Fabs were expressed in culture volumes of 100–300 ml depending on expression levels. Bacteria were grown at 37°C in 2× YT broth supplemented with 100 μg/ml carbenicillin and 0.1% glucose until the A_{600} value reached ~1.3, after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and the culture was continued overnight at 30°C. Bacteria were pelleted by centrifugation and the periplasmic fraction was extracted by resuspending the pellet in 1–3 ml (depending on

Cluster 2

Heavy chain	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3-21 JH6	SGGGLVKPGGSLRLSCAAS	GFTFSSYS	MNWVRQAPGKGLEWVSS	ISSSSSYI	YYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYC		WGQGTTVTVS
2-152A11	...D.....EV.	.....T	.....	...T....	.....S.....I...	A...T...	.....L....
1-61C04	...D.....E..	.....RFK	.....	V..T.A..	D..A.....L.....T....S.H....A.....	.T.....	.....
1-63D10	...D.....E..	.....RFK	.....	V..T.A..	D..A.....T....S.H....A.....	.T.....	.....L....
2-135D08	.....A.....VV.	...L...K	.....	...T.A..	D.....E.S....T.S.....	.....	.....L....
2-152B08	.....VV.	...L...K	.....	...T.A..	D.....E.S....T.S.....	.....	.....L....
2-152B10	.....VV.	...L...K	.....	...T.A..	D.....A.....E.S....T.S.....	.....	.....L....
2-152D10	.....VV.	...L...K	.....	...T.A..	D.....E.S....T.S.....	.....	.....L....
1-63B07	.....V.	....NNFR	.....A...D.A..	...T.A..	D.....R.....T.....R..V...G....	.....F..	.....L....
2-152C04	.....	..A..N.K	.....S.....A.	...RNTH.	F..N.....S.....DT.....L...	.T...H.F	.....L....
Cluster CDR3 consensus						VSGSSLDY	

Light chain	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VK1-39 JK1	SPSSLSASVGDRVTITCRAS	QSISSY	LNWYQQKPGKAPKLLIY	AAS	SLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	T	FGQGTKVEIK
2-152A11	.....I.....	...NTN	.....F	...	N.....E.S...T.....	..N.I....	.....
1-61C04	.....Y.....V.....	....TN	...F..Q.....Q....	SS.	T..T.....A....S..N.....	.....VI..	.....QA.
1-63D10	.....Y.....	....TN	.S.L..Q.....Q....	SS.	T..T.....S..N.....	.....VI..	.....QA.
2-135D08	..P.....	....TN	...F.....N....	S..	T..T.....	.....	.....R
2-152B08	..P.....	....TN	...F.....N....	S..	T..A.....	.....	.....R
2-152B10	..P.....	....TN	...F.....N....	S..	T..T.....	.....	.....R
2-152D10	..P.....	....TN	...F.....N....	S..	T..T.....	.....	.....R
1-63B07	.....A....	....TN	...F..T.....F	S..	T..R.....E.....	.....SH..	.....D..
2-152C04	.....	....TN	.....R.....I...F	...	N..T.....	Q....DI..	.....M...
Cluster CDR3 consensus						LQTYSPRR	

FIGURE 3. Alignment of cluster 2 V_H and V_κ gene amino acid sequences. Sequences are represented as in Fig. 2.

culture volume) of ice-cold PBS containing 0.94 M NaCl and 0.8 mM EDTA. Fabs were further purified on protein L spin columns according to the manufacturer's instructions (Pierce). The concentration of Fab was determined by an indirect immunoassay using a purified Fab as standard. SPR analysis was performed using a Biacore 2000 apparatus (Biacore AB). TT was immobilized on a CM5 chip surface using standard amine coupling chemistry to a level resulting in a maximum response unit (RU) value of ~100 RU or less. Purified Ab Fab were diluted serially in HBS-EP running-buffer (10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20; Biacore) and passed over the chip at 50 μl/min. All measurements were conducted at 25°C. In most cases, association was measured for 5 min and dissociation for 20 min. However, for Fab with very slow off-rates (below $\sim 1 \times 10^{-4}$ s^{-1}), dissociation was measured for longer periods of time, up to 16 h, depending on the off-rate. Rate constants (k_{on} and k_{off}) and affinity constants (K_D) were determined using the BIAevaluation software (Biacore) by the global fitting of 4–6 different concentrations passed over the same sensor surface. Global fits of k_{on} and k_{off} simultaneously gave average χ^2 values of 1.8 and 0.8, respectively.

Results

Isolation of Ab repertoires

Ab repertoires were, as reported previously (10), made from plasma cells obtained from blood of two healthy volunteers (donors TT01 and TT02) after a boost with TT. Using the Symplex Technology, which involves high throughput single-cell sorting, single-cell RT-PCR, and bulk cloning of the isolated IgG H chain and κ L chain variable region gene pairs (V_H-V_κ), repertoires were generated with preservation of the cognate H and L chain pairing. These repertoires were subsequently expressed in bacteria and screened for Ag reactivity. This led to the isolation of 122 and 84 unique tetanus-specific V_H-V_κ gene products from donor TT01 and TT02, respectively.

Statistical analysis of repertoire sizes

Based on *V* and *J* gene segment usage and CDR3 sequence homology, we estimated the numbers of rearrangement events in the isolated Ab repertoires to be 29 and 40 events for donor TT01 and TT02, respectively. Because the number of isolated sequences for each donor is much larger than the number of rearrangements (four and two times larger for TT01 and TT02, respectively), it appears reasonable to make statistical predictions of actual repertoire sizes based on the isolated libraries. The analysis is based on the assumption that no significant skewing of the data set by nonrandom gene segment usage occurred (12). It calculates the statistical number of distinct rearrangements (the statistical/theoretical repertoire size) from the number of examined sequences and the number of rearrangements in the data set. This analysis returned statistical maximum likelihood estimates of the repertoire size of 29 and 48 distinct rearrangement events for donor TT01 and TT02, respectively. Ninety-five percent one-sided confidences were calculated as the smallest number of rearrangements for which the probability of observing the distinct number of gene rearrangements among the number of examined sequences was <5%. These confidences returned values of 31 and 57 rearrangement events for donors TT01 and TT02, respectively.

Distribution of variable gene fragment usage in human anti-TT Ab repertoires

Major structural restrictions of an Ab response can be identified by a genetic analysis of the variable region gene segment usage. Hence, the distributions of *V* gene segments and *J* gene segments for the V_H and V_κ genes of donor TT01 and TT02 were analyzed and compared with a naive repertoire from peripheral IgM$^+$ B cells (13, 14) (Fig. 1). Both TT repertoires showed extensive diversity in their *V* gene segment usage. The five most prevalent H chain V gene segment families were found with only minor differences relative to the naive repertoire (Fig. 1*A*). The H chain *J* gene segment usage also followed the naive repertoire except for the J_H5 gene segments, which were less frequently used (Fig. 1*B*). The distribution of κ-chain *V* gene families followed the naive repertoire dominated by the $V_\kappa1$ and $V_\kappa3$ families, although the $V_\kappa2$ gene family was less prevalent in the TT repertoires (Fig. 1*C*). Similarly, κ-chain *J* gene segment usage showed a similar distribution between the repertoires except for $J_\kappa5$ which was less frequent in the TT01 repertoire (Fig. 1*D*). Thus, the TT repertoires showed extensive diversity without any major biases in *V* and *J* gene segment usage.

Distribution of H chain CDR3 lengths

Binding site topology is highly dependent on the length and composition of the CDR3 of the H chain. Hence, restricted Ab responses have been shown to select for CDR3 loops of particular lengths (15–17). Fig. 1*E* shows the distributions of the CDR3 lengths of donor TT01 and TT02 in comparison to a randomly selected set of Abs with different specificities (18) and a highly restricted anti-rhesus D Ab repertoire (15). The distribution of the randomly selected Ab repertoire is relatively broad, going from 3 to 27 aa with an average CDR3 length of 14. In marked contrast,

Table I. *Kinetics and affinity constants of Abs with deduced germline variable region genes and their hypermutated variants and the influence of hypermutations on Ab binding*

Rearrangement cluster[a]	Clone	On-Rate (M^{-1} s^{-1})	Off-Rate (s^{-1})	$t_{1/2}$ (min^{-1})	Affinity (M)	No. of Hypermutations (V_H + V_κ)
1	Germline V_H1–69-J_H6 $V_\kappa1$–27-$J_\kappa3$	3.0×10^6	3.3×10^{-4}	35	1.1×10^{-10}	0 + 0
	60D05 (V_H1–69-J_H6 $V_\kappa1$–27-$J_\kappa3$)	6.4×10^6	1.7×10^{-5}	670	2.7×10^{-12}	13 + 6
	62D09 (V_H1–69-J_H6 $V_\kappa1$–27-$J_\kappa3$)	2.7×10^6	5.6×10^{-5}	210	2.1×10^{-11}	10 + 6
	63B12 (V_H1–69-J_H6 $V_\kappa1$–27-$J_\kappa3$)	4.6×10^6	7.4×10^{-5}	160	1.6×10^{-11}	10 + 4
2	Germline V_H3–21-J_H6 $V_\kappa1$–39-$J_\kappa1$	6.8×10^5	1.7×10^{-1}	0.1	2.5×10^{-7}	0 + 0
	63D10 (V_H3–21-J_H6 $V_\kappa1$–39-$J_\kappa1$)	1.0×10^5	4.2×10^{-4}	28	4.0×10^{-9}	14 + 13
	60A02 (V_H3–21-J_H3 $V_\kappa1$–27-$J_\kappa2$)	9.1×10^3	1.3×10^{-4}	90	1.4×10^{-8}	0 + 0

[a] Related to cluster numbers in Figs. 2 and 3.

the highly restricted rhesus D Ab repertoire shows a narrow distribution ranging from 14 to 30 aa with a prominent peak of ~18 aa. The CDR3 length distributions of donor TT01 and TT02 are both broad, ranging from 8 to 26 aa, indicative of an unrestricted response. However, both TT repertoires showed an increased preference for longer CDR3 lengths of ~15 or 16 aa, leading to averages of 17 and 16 aa, respectively, which was higher than the unselected repertoire. Thus, although the TT responses were diverse in their requirement for CDR3 lengths, there was a bias toward more extensive contact regions.

Sequence homology among Abs within and between anti-TT repertoires

Structurally restricted Ab responses are known to lead to almost identical Abs from different individuals (15, 16). To investigate whether any of the *V* gene diversity was shared between the two donors, a multiple alignment was performed on the combined set of 206 V_H and V_κ sequences (data not shown). *V* gene sequences of the two donors were evenly distributed throughout the cladogram. This indicates that both donor repertoires possessed extensive diversity with no dominating preferences for particular *V* genes in agreement with the previous analyses. However, two clusters of homology contained *V* gene sequences from both the TT01 and TT02 repertoires. The V_H and V_κ sequences were aligned separately for each of the two clusters. Abs belonging to cluster 1 (Fig. 2) all had H chain CDR3 lengths of 15 aa containing the LGGTR consensus motif or single amino acid variants thereof. There was an exclusive usage of the J_H6 gene segment and the *V* gene alleles V_H1-69 or V_H3-21. The κ-chain *V* gene usage was restricted to the alleles V_κ1-27 and V_κ3-20, whereas CDR3 length varied from 9 to 10 aa and κ-chain *J* usage involved $J_\kappa1$, $J_\kappa2$, and $J_\kappa3$. In total, the different H and L chain rearrangements made up

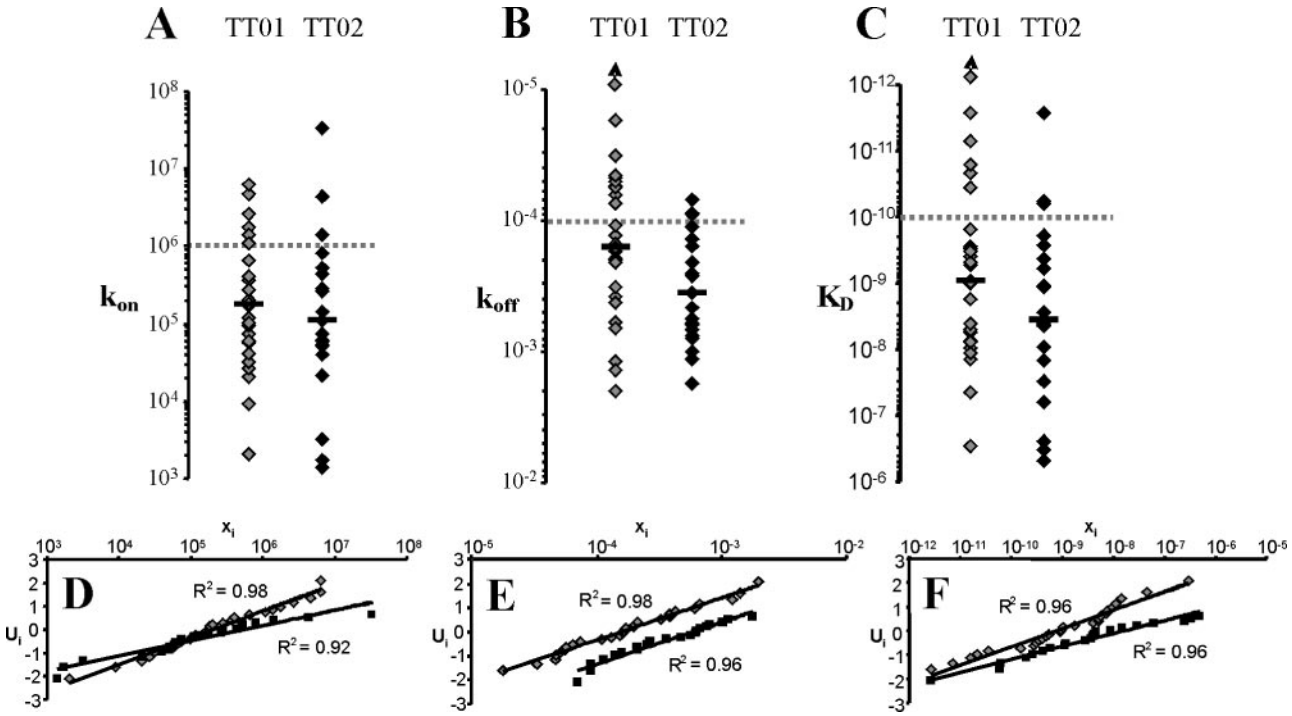

FIGURE 4. Binding constant distributions. *A–C*, Distributions of on-rates (*A*), off-rates (*B*), and affinity constants (*C*) of anti-TT Fab. Data originating from donor TT01 (n = 28) are indicated by gray dots whereas data from donor TT02 (n = 21) are indicated by black dots. The black bars show the median of each data set. Arrows indicate that the dissociation of this particular Ab Fab (clone 60F01) was too slow to measure accurately using SPR, hence only giving an upper limit for off-rate and affinity. Dashed gray lines indicate theoretical limits proposed by Foote and Eisen (7). *D–F*, Normal fractile plots of on-rates, off-rates, and affinity. The horizontal axis presents kinetic values (x_i) whereas the vertical axis indicates normal fractiles (U_i). Clone 60F01 was left out of the distribution analyses of k_{off} (*E*) and K_D (*F*).

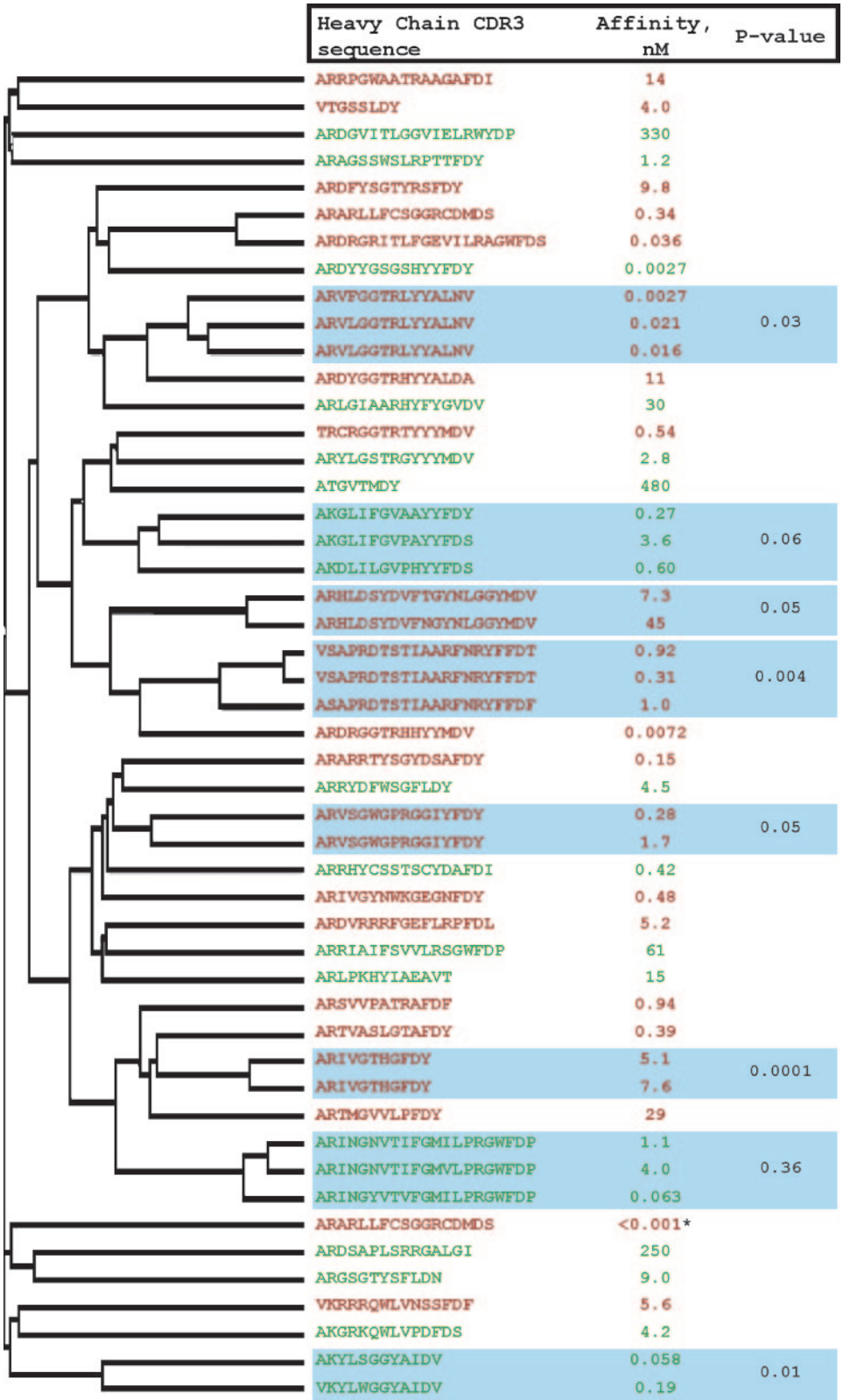

FIGURE 5. Relationship between genetic and functional diversity. *Left-hand column* shows H chain CDR3 sequences ordered by a cladogram (red, TT01; green, TT02). *Middle column* presents the affinity in nanomolar measured for each Fab. Clonally related Abs are marked by blue boxes. The affinity of the Ab marked by an asterisk (*; belonging to clone 60F01) could not be measured precisely because it has a very slow off-rate that is beyond the detection limits of SPR. The affinity was estimated to be <1 pM. Difference in variance of affinities was tested by a two-sided *F* test on variance in affinities within each of the eight groups of clonally related Abs against the variance of all measured affinities. *p* values returned by the test are shown in the *right-hand column* for each of the eight groups.

five different assemblies of Ab germline genes. Competitive binding studies among Abs belonging to cluster 1 by SPR revealed that they all recognized overlapping epitopes (data not shown). Abs using the V_H1-69 all originated from the TT01 repertoire and could therefore potentially be of common clonal origin, and their differential usage of three different V_κ genes could be explained by receptor revision (19). However, the Abs appeared to be the result of independent rearrangement events because only few somatic mutations were shared among the V_H genes, which contrasts with previous examples of receptor revision of Ab L chains (20), and because differences within CDR3 were mainly located in the H chain *V-D* and *D-J* joining regions, which are encoded by random insertion/deletion of nucleic acids and thus typically unique for each clonal rearrangement. An analysis of silent mutations confirmed

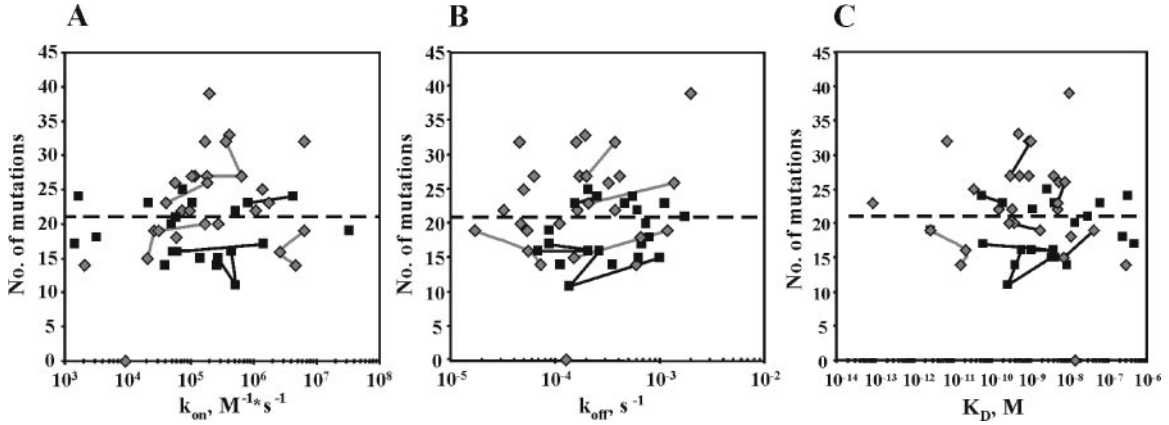

FIGURE 6. Correlation between number of somatic mutations and binding constants in terms of on-rate (*A*), off-rate (*B*), and binding affinity (*C*). Gray diamonds represent Abs from donor TT01 ($n = 28$ for k_{on} and $n = 27$ for k_{off} and K_D) whereas filled squares represent those from donor TT02 ($n = 21$). Clonally related Abs (marked by blue boxes in Fig. 5) are connected by lines. The punctured horizontal line indicates the average number of hypermutations per V_H-V_κ gene pair.

these observations (not shown). Selection of such highly homologous albeit clonally independent Abs was further demonstrated by the remaining Abs belonging to cluster 1, which were isolated from both the TT01 and the TT02 repertoires. Thus, it appeared that Abs from TT01 belonging to cluster 1 were the result of four independent rearrangement events rather than being the result of receptor revision of a single B cell progenitor. Cluster 2 (Fig. 3) consisted of Abs from the TT01 and TT02 repertoire characterized by having H chain CDR3 lengths of 8 aa containing the (S/T)SG(S/T)L motif and L chain CDR3 lengths of 9 aa containing a central QTYS consensus motif. There was an exclusive usage of the *V_H3-21* allele together with *J_H6* paired with a L chain having the *Vκ1-39* allele joined to *J_κ3*. Furthermore, several somatic mutations were shared between the Abs independently of donor origin. This further demonstrated the selection of highly homologous albeit clonally independent Abs. Together, these observations indicated that structural restrictions were indeed shaping parts of the Ab repertoire against TT despite the extensive overall diversity of the TT01 and TT02 repertoires and the vast complexity of the antigenic surface.

Binding characteristics of Abs in germline configuration

To investigate whether the Abs of cluster 1 and 2 originated from germline Abs of particularly high affinity, Fab genes were constructed in their deduced germline configuration and their binding constants were determined by SPR (Table I). Cluster 1 appeared to originate from a germline Ab of fast association and slow dissociation leading to high affinity, whereas cluster 2 originated from an Ab of fast association and fast disassociation leading to a relatively modest affinity. One Ab, 60A02, isolated from TT01, was without any identifiable hypermutations and had a modest on-rate and a slow off-rate leading to high affinity, although not as high as that seen for cluster 1. These observations indicate that Abs of the primary repertoire can be of high affinity and have interactions of relatively high stability. Further, comparison of the binding constants of the hypermutated Abs with their deduced germline counterparts revealed that the positive effects of hypermutations were mainly acting by lowering the off-rates whereas the on-rates were both positively and negatively affected by the hypermutations. Moreover, in both cluster 1 and 2 hypermutations lead to an ~100-fold increase in affinity.

Distribution of affinity and binding rate constants

To address the proposed limits for Ab affinity and kinetics, we determined the binding rate constants of representative sets of Abs from the TT01 and TT02 repertoires. The association rate (k_{on}), dissociation rate (k_{off}), and affinity constant (K_D) values of 28 TT01 and 21 TT02 Fabs were determined by SPR (Fig. 4, *A–C*). Association rates showed a wide range of distributions covering almost 5 orders of magnitude. The medians for the TT01 and TT02 repertoires were of similar magnitude at 1.77×10^5 M^{-1} s^{-1} and 1.10×10^5 M^{-1} s^{-1}, respectively. The dissociation rates of TT01 ranged >2 orders of magnitude with a median value of 1.6×10^{-4} s^{-1} whereas the TT02 repertoire was more restricted covering, 1.5 orders of magnitude with a median value of 3.6×10^{-4} s^{-1}. Affinity constants showed the widest range covering up to 6 orders of magnitude with median values of 9.3×10^{-10} M and 3.6×10^{-9} M for the TT01 and TT02 repertoires, respectively. Thus, affinities ranged from micromolar to picomolar values and the median affinity of the TT01 repertoire was 4-fold higher than the median of the TT02 repertoire due to a ~2-fold difference in both k_{on} and k_{off} rate medians. To examine whether binding constants were randomly distributed, data sets were plotted as logarithmic normal fractile diagrams (Fig. 4, *D–F*). All data sets fitted well to linear regressions, with R^2 ranging from 0.91 to 0.99 as expected for a log-normal distribution. Hence, no obvious biases were apparent and the data sets seemed evenly distributed around the median as expected for a random distribution.

The relationship between V gene homology and affinity

To investigate the relations between genetic homology and affinity, V_H and V_κ sequences of Abs with known affinity were combined into one single continuous sequence for each Ab and aligned according to homology. The affinity constants were evenly distributed throughout the cladogram (Fig. 5), indicating no general correlation between affinity and *V* gene composition. However, the affinity between clonally related Abs varied in the order of 10- to 100-fold, which was significantly less than the 10^6-fold variation observed for the whole set.

Relationship between number of somatic mutations, affinity, and kinetics

The relationship between somatic amino acid replacements and binding constants was investigated by plotting for each TT repertoire the combined number of replacements in the V_H and V_κ genes against k_{on}, k_{off}, and K_D (Fig. 6, *A–C*). The average number of replacements for the full data set was 21 per *V* gene pair, whereas the average number for donor TT01 and donor TT02 separately

were 23 and 19, respectively. The scattered distributions of the data points on all plots indicated that within each repertoire there was no correlation between the number of amino acid replacements and the binding strength. This was also the case among clonally related Abs. Plotting replacement frequencies of V_H and V_κ separately did not change the appearance of the data (not shown). Hence, somatic mutations had multiple effects on Ag binding, reflecting the intrinsic properties of random somatic diversification. Regardless, the higher replacement average of TT01 relative to TT02 correlated with the increased average in binding strength, supporting the notion that somatic mutations, together with Ag selection, lead to enhancement of the overall affinity of Ab repertoires.

Discussion

To determine the diversity, affinity, and kinetic limits for the human polyclonal Ab response we have examined the genetic diversity and binding characteristics of comprehensive panels of mAbs obtained from two individuals immunized with TT. The Ab repertoires were generated using a novel technique for the efficient cloning of cognate pairs of human Ab V genes from single plasma cells, the Symplex Technology (10). These repertoires consisted of 122 and 84 unique tetanus-specific *V* gene pairs and can thus be considered the most authentic Ag-specific Ab repertoires of single individuals reported to date.

The two TT repertoires showed extensive diversity in their *V* gene family and *J* gene segment usage for the V_H and V_κ genes without any major biases toward certain gene segments. The extent of the diversity was similar to a naive repertoire from peripheral IgM^+ B cells (13, 14) and thus matched the extensive structural complexity of the TT surface, which creates a vast number of putative Ab epitopes. The TT responses were also diverse in their requirement for V_H-CDR3 lengths, although there was a bias toward a more extensive CDR3 relative to a randomly selected repertoire of different specificities (18). This concurs with a previous report, which showed that secondary response repertoires on average use longer CDR3 than primary repertoires (21), leading to the hypothesis that large Ags may skew the V_H repertoire toward longer CDR3 loops by allowing more contact points and, hence, higher affinity for the Ab combining site.

Despite the extensive genetic diversity found within each repertoire, we found two shared clusters of highly homologous *V* genes. Within each cluster, members were characterized by CDR3 consensus motifs, restricted *V* and *J* gene segment usage, and characteristic amino acid replacements. Further, within the TT01 repertoire it appeared that highly homologous albeit clonally unrelated Abs were selected from the naive repertoire. Thus, despite the vast complexity of the antigenic surface of TT there was a common preference for certain *V* gene configurations. To test whether this was caused by high native affinity of these particular rearrangement events, *V* gene pairs of each cluster were synthesized in their proposed germline configuration and expressed as functional proteins. The affinity constants (K_D) of cluster 1 and 2 germline Ab Fabs were 1.1×10^{-10} M and 2.7×10^{-7} M, i.e., of high and intermediate affinity, respectively (Table I). Thus, selection of these highly homologous *V* gene sequences appears to be caused by their relatively high native affinity for TT. Further, we found one Ab without somatic mutations in the TT01 repertoire with a K_D of 1.4×10^{-8} M. Although this Ab did not belong to any of the shared clusters, it confirmed the possibility of naive Abs of high affinity from primary repertoires. This raises the question of whether such naive high affinity Abs are due to the evolutionary selection of certain innate *V* genes matching common pathogens (in this case tetanus toxin) or whether it simply reflects a random occurrence of rare high-affinity B cell clones within the naive repertoire. The latter possibility appears the most likely, because high-affinity Abs without hypermutations have also been observed in human responses against the rhesus D Ag (15), in short term responses in mice toward lysozyme (6, 22), and in mice responding toward a nonpathological viral infection (5).

Selection of high-affinity Abs in vitro from naive phage display libraries of 10^{10} clones or greater has resulted in Ab affinities in the subnanomolar range (23). Considering that the total number of naive B lymphocytes in the human organism is $>10^{10}$, it appears reasonable to expect a similar selection of such high affinity Abs from the primary repertoire in vivo. Further considering the occurrence of naive high-affinity Abs a purely statistical event, it follows that structurally complex Ags harboring an exhaustive set of epitopes are more likely to select for early high-affinity Abs relative to less complex Ags harboring only a few epitopes. Such an argument would explain the lack of early high-affinity Abs in the first reports on anti-hapten responses in mice that contrasts the later observations on structurally more complex protein Ags. Alternatively, the absence of early high-affinity Abs in responses against haptens can be explained by the multivalency of the Ag when coupled to the carrier protein, which contrasts the monovalency of the protein Ags mentioned above. Hence, anti-hapten responses would be dominated by avidity effects favoring the selection of low-affinity Abs in the primary response (24).

Based on *V* gene sequence homology, we have estimated the number of clonal selection events creating the two isolated anti-TT IgG/κ Ab repertoires to be 29 and 40 events, respectively. These repertoires can be considered representative of the native Ag-selected repertoires from the two donors for several reasons. Firstly, the isolation of clonally related Abs within a repertoire indicated good sampling of the existing repertoire. Secondly, the isolation of highly homologous albeit clonally unrelated Abs within a repertoire as well as between repertoires further indicated good sampling of the actual repertoires. Third, phage display on combinatorial libraries that was made using total RNA from TT01 blood cells (our unpublished data) gave, in 95% of the cases, V_H rearrangements identical to those identified with Symplex. Statistical analysis of the data set of each donor returned 95% confidences at 31 and 57 rearrangement events for the IgG/κ Ab repertoires of donor TT01 and TT02, respectively. However, actual repertoire sizes are likely to be somewhat larger than estimated by pure statistics because the statistical analysis is based on the assumption that all clones are equally represented within the repertoire. Thus, further assuming that the Vλ repertoire, which typically constitutes 40% of a human Ab response, is of equal complexity, the Ag-selected repertoires against TT is estimated to be in the order of 100 clonally unique Abs in addition to hypermutated variants thereof.

Dissociation and association rate constants of biologically active molecules are normally in the range of 10^0 to 10^{-5} M^{-1} s^{-1} and 10^{-2} to 10^{-7} M^{-1} s^{-1}, respectively. Low affinity receptors are characterized by having slow on-rates in the range of 10^2 to 10^4 M^{-1} s^{-1} and/or fast off-rates in the range of 10^0 to 10^{-2} s^{-1}, whereas high affinity receptors are characterized by having fast on-rates in the range of 10^5 to 10^7 M^{-1} s^{-1} and/or slow off-rates in the range of 10^{-3} to 10^{-5} s^{-1}. Both TT repertoires essentially cover the entire biological range of k_{on} values, indicating no special preference for a particular k_{on}. In contrast, the k_{off} range of both repertoires had a lower limit of 2×10^{-3} s^{-1}, which is well beyond what is generally found for low-affinity ligands. Even though we found examples of unmutated Abs of high intrinsic affinity, it must be assumed that most of the Abs were initially of relatively low affinity (and fast dissociation) as exemplified by the

fast dissociation rate of cluster 2 in germline configuration. Further, the ELISA used for the screening and selection of clones for this study were able to identify TT Abs with fast dissociation kinetics from phage display libraries (our unpublished results), verifying that the observed lower limit for dissociation is not a consequence of technical limitations. Thus, it appears that the off-rate is subjected to more stringent requirements for efficient clonal expansion and selection than the corresponding on-rate. Differential effects of the two kinetic parameters on clonal selection have previously been observed (2, 24, 25) and are also the rationale behind the proposed affinity limits for the maturing Ab response (7). Although studies using B and T cell hybridomas have demonstrated that Ab-mediated Ag presentation requires an off-rate of at least $\sim$0.5 M s^{-1} (25), our findings suggest that this limit is moved a further 250-fold to 2×10^{-3} s^{-1} in order for efficient clonal expansion and selection in a developing Ab response in vivo. Interestingly, this minimum half-life of Ab/Ag interactions of 5.7 min corresponds to the estimated half-life of 8 min for membrane-bound IgG, which offers an explanation for the requirement for high affinity for the Ab-mediated internalization of monovalent and soluble Ag by specific B cells for presentation to T cells at the beginning of a T cell-dependent humoral response (24). Further emphasizing the importance of off-rates on clonal selection, we found that the affinity maturation due to somatic mutations within cluster 1 and 2 mainly affected dissociation with little or even negative effect on association.

The even distribution of affinities and kinetic rate constants around their medians follows a log-normal distribution. This concurs with the random nature of somatic rearrangement and hypermutation events and is in agreement with clonal selection being based solely on ligand binding characteristics. Median values were all close to the proposed theoretical limits, which further supports the existence of an affinity ceiling based on positive selection. Because both repertoires yielded similar median values and showed extensive diversification by somatic mutation as expected for highly matured Ab repertoires, it appears reasonable to make predictions regarding the kinetic and affinity limits for positive selection of B lymphocytes: association rates accumulated at 10^5 and dissociation rates at 10^{-4}, leading to an affinity ceiling of $\sim 10^{-9}$ M. These predictions are supported by a previous report finding a similar affinity ceiling of 0.5×10^{-9} M in Ab repertoires from mice responding to a viral infection (5). However, the affinity ceiling does not appear discrete, because evidence for affinity maturation beyond 10^{-10} M was observed within cluster 1. Thus, the possibility remains that the ceiling is at an even higher affinity than suggested. One such additional factor that could push the affinity ceiling even further into the lower picomolar range could be interclonal competition for limited amounts of Ag (25).

Both repertoires were extensively mutated, indicative of a highly matured response. Within each repertoire it was not possible to observe any correlation between binding strength and level of amino acid replacements, even among clusters of Abs of identical clonal origin, indicating that the repertoire was saturated in terms of somatic alterations. The only positive correlation between the level of hypermutation and affinity was between the two repertoires. TT01 had a median affinity 4-fold above that of TT02, which correlated to the averages of 23 and 21 aa transitions per *V* gene pair, respectively. Thus, even though both repertoires were saturated with hypermutations, the overall effect on affinity was still positive.

The observation that for germline Abs of clusters 1 and 2 affinity maturation led to maximally a 100-fold increase in affinity and the observation that Ab affinity was generally restricted to vary 10- to 100-fold among clonally related Abs point to the possibility that each rearrangement carries a limited potential in regard to affinity maturation. This is in agreement with previous observations in which secondary responses of enhanced affinity involved changes in *V* gene usage (2) and also in agreement with the limitations on affinity maturation imposed by particular amino acid replacements in developing anti-hapten Ab responses in mice (26). It further implies that the large affinity range spanning six logarithmic units, observed for both TT repertoires, is mainly due to differences in native affinity among the Abs selected from the primary repertoire. An extensive range of affinities in the primary repertoire would also explain the observation of early high affinity Abs as discussed above.

We here present a molecular analysis of two human Ab responses against TT. Each repertoire is composed of extensive genetic diversity involving most *V* gene families and *J* gene segments. The repertoires appear unrestricted and representative, and the human polyclonal repertoire can thus be estimated to be in the order of 100 clonal selection events. Affinity and association constants are evenly distributed over a relatively large range, whereas dissociation rates cover a more restricted range of relatively slow dissociation. Further, we find evidence that affinity maturation mainly favors reduction in dissociation rates. Hence, the dissociation and association rates have a differential effect on the developing Ab response, with more strict requirements for the dissociation rates. Finally, identification of high-affinity Abs without hypermutations in combination with a relatively modest effect of hypermutations on affinity increases indicates that the primary repertoire has a relatively large affinity span into the subnanomolar range. Taken together, our results have implications for the understanding of the development of human Ab responses and for the understanding of polyclonality in an immunological context.

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Disclosures

All authors are employees (with equity interests) of Symphogen, a biotech company that is developing recombinant therapeutic polyclonal antibodies.

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Paper 2

Title:

Limits for affinity maturation and repertoire diversification in human antibody responses towards antigen

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The immune system is known to generate a diverse panel of high affinity antibodies by adaptively improving the recognition of pathogens during ongoing immune responses. We here report the biological limits for antigen-driven affinity maturation and repertoire diversification by analyzing antibody repertoires in two adult volunteers after each of three consecutive booster vaccinations with tetanus toxoid. Maturation of on- and off-rates occurred independently, indicating a kinetically controlled affinity maturation process. The third vaccination induced no significant changes in the distribution of somatic mutations and binding rate constants implying that the limits for affinity maturation and repertoire diversification had been reached. These fully matured antibody repertoires remained similar in size, genetically diverse and dynamic. Somatic mutations and kinetic rate constants showed normal and log-normal distribution profiles, respectively. Mean values can therefore be considered as biological constants defining the observed boundaries. The mean level of somatic mutations was saturated at 13.7 and 7.4 amino acid transitions per V_H and V_K gene segment, respectively. At physiological temperature, affinity maturation peaked at $k_{on}=1.6x10^{4}M^{-1}s^{-1}$ and $k_{off}=1.7x10^{-4}s^{-1}$ leading to a maximum mean affinity of $K_D=1.0x10^{-9}M$. At ambient temperature, the average affinity increased to $K_D=3.4x10^{-10}M$ mainly due to slower off-rates. This experimentally determined set of constants can be used as a benchmark when analyzing the maturation level of human antibody responses.

When challenged with a foreign substance the immune system mounts a response of specific antibodies, which initially are of predominantly low to intermediate binding strength [1-3]. Binding strength of subsequent generations of antibodies is improved by introducing mutations in the V regions and the resulting variants are selected on the basis of binding to the antigen [2-4]. Kinetic boundaries for such maturation of antibody affinity have been proposed [5,6]. By considering physiochemical limits for association rates (k_{on}) and the rate of receptor internalization upon antigen binding as limiting dissociation rate (k_{off}) maturation, Foote and Eisen predicted a maximum natural k_{on} in the range of 10^5-$10^6 M^{-1}s^{-1}$ and minimal natural k_{off} in the range of 10^{-3}-$10^{-4}s^{-1}$ leading to a natural affinity ceiling with equilibrium dissociation constants (K_D) in the order of 10^{-8}-10^{-10}M. These theoretical boundaries have been difficult to verify experimentally, due to the inability of existing antibody cloning techniques to generate a sufficient number of truly representative antibody clones from single repertoires of animal or human origin. Recent advances in the study of single B cells has eliminated this limitation and allowed for extremely detailed studies of the composition and functionality of antigen-driven antibody repertoires at any given time point [7-12].

Herein, we determine experimentally the ceiling for natural affinity maturation and the limits for genetic diversification of human antibody repertoires developing over time in response to a single antigen. Antibody responses were followed in two human volunteers subjected to three consecutive boosts with the standard tetanus toxoid (TT) vaccine. Vaccinations were separated by at least 1.5 years and blood was drawn for analysis 6 days after each challenge, at which time point the circulating antibody secreting cells specific for TT are known to peak [13,14].

Using the Symplex™ technology [15], antibody repertoires were prepared from circulating plasma blasts separately after each challenge. Between 2100-3600 single clones from each of the repertoires were expressed as Fab fragments and screened for TT binding and specific antibodies were picked for DNA sequencing and functional characterization (table 1). As previously described [11], V and J gene segment usage and somatic mutations were determined by aligning V_H and V_L gene sequences to the IMGT database [16].

The amino acid sequences of 594 unique TT-specific antibody clones were analyzed, and estimated to originate from at least 195 different naïve B cell progenitors (termed clonotypes). The entire antibody panel was highly diverse and contained germline gene segments from all the major families of V_H, J_H, V_K and J_K except the rarely found V_K6 (see supplementary data).

Despite repeated antigen challenge and consequently consecutive rounds of somatic mutations and affinity selection, the overall number of somatic mutations present in each repertoire remained constant for each donor (figure 1A). Thus, the maximum tolerated level of somatic mutations appeared to have been reached. Statistical analysis of the combined distribution of amino acid somatic mutations in all six repertoires indicate a normal distribution around clearly defined mean values of 13.7, 7.4, and 21.1 for V_H, V_L and the combined repertoire, respectively. Previous reports on somatic mutations [7,8,17-19] fall within the presently observed limits, which can thus be regarded as representative for a fully matured response.

The number of unique clonotypes identified in each repertoire was between 29 and 67 (table 1) and maximum likelihood estimates of the overall repertoire sizes were determined as previously reported [11,20] (figure 1B). It appears that antigen-induced human IgH-V_K repertoires do not to exceed in the order of 100 clonotypes. As the antigen is not imposing any restrictions to the observed responses, the observed limitation in repertoire sizes is likely to be intrinsic to the immune system when responding to large, structurally complex antigens [12,15,18,21]. Approximately two thirds of the clonotypes in the TT2 repertoires were found associated with only a single vaccination event. And this fraction also increased for TT1 repertoires as vaccinations progressed (Figure 1B). Further, several clonotypes unique to the TT1 repertoires carried no somatic mutations, notably after the second booster vaccination. Together, this suggest a constant influx of new clonotypes from the naïve repertoire to an already existing repertoire. So despite repeated exposure, antibody responses against complex antigens remain diverse and dynamic rather than converge towards a few clonotypes with optimal binding properties, as it has been reported for hapten-driven responses [22-25].

To determine the progression in affinity maturation, k_{on} and k_{off} were determined at 25°C for a genetically diverse set of 19-35 antibodies from each repertoire (figure 2).

The differences between the six repertoires in distributions of k_{on} values were not statistically significant, indicating that the ceiling for maturation of k_{on} had already been reached prior to the first booster vaccinations of this study, in accordance with the fact that the Danish child vaccination program involves at least four tetanus immunizations. To quantify the limit for affinity maturation of k_{on}, k_{off} and K_D, selected data sets were combined and analyzed statistically (figure 3A-C; Table 2). The joint distribution of k_{on} appeared log-normal with a geometric mean of $2.0x10^5M^{-1}s^{-1}$. For both donors, repertoires from the first vaccination had significantly faster k_{off} (TT1: $p=0.001$; TT2: $p<0.001$) indicating that the limit for maturation of k_{off} was first reached after the second vaccination. Thus, to quantify the limit for affinity maturation of k_{off}, the four data sets from the last two vaccinations were combined for statistical analysis. They showed a clear log-normal distribution with a geometric mean at $7.0x10^{-5}s^{-1}$. As a consequence of the relatively dispersed and fully matured k_{on}, differences between repertoires were insignificant for K_D. The combined average K_D from the second and third vaccinations was determined to be $3.4x10^{-10}M$. The log-normal distribution of binding constants is in accord with their logarithmic relationship with the Gibbs free binding energy. Multiplicative standard deviations ranged from 3.7 to 8.6 indicating strongly skewed distributions compared to log-normal distributions in other biological systems, where coefficients usually range from 1.1 to 3.2[26]. The observed separate maturation of kinetic rate constants supports that maturation of antibody responses is kinetically rather than thermodynamically controlled[1,2,27].

To determine the limits for affinity maturation at physiologically relevant temperature, the rate constants were determined at 37°C for a representative set of 50 antibodies from the last two vaccinations from both donors. As observed for measurements at ambient temperature, data was log-normally distributed around clearly defined mean values (Figure 4A-D). The geometric mean for k_{on} was not affected by the change in temperature. In contrast, the k_{off} geometric mean value increased 2.4-fold to $1.7x10^{-4}s^{-1}$ which corresponds to a drop in average interaction half life from 165 min. to 70 min. when binding at physiological temperature. Combined this gave a 3.0-fold change in the mean K_D at $1.0x10^{-9}M$ (Table 2).

In summary, to determine the natural boundaries for maturation of human antibody repertoires in response to antigen, Symplex™ repertoires were made from two human donors after each of three booster vaccinations with TT. Analysis of the genetic diversity, somatic maturations, and kinetic rate constants revealed that despite being saturated in terms of binding and somatic mutations, repertoires remained genetically dynamic and diverse. The most likely immunological significance of this dynamic behavior is that it provides an efficient countermeasure to the constant genetic evolution of pathogenic microorganisms. Finally, we provide the first experimental determination of the limits for antibody affinity maturation and repertoire diversification in single individuals in response repeated vaccinations. The identified kinetic mean constants are in accordance with the theoretical ranges proposed by Foote & Eisen [5] although the relatively long mean half-life at physiological temperature of 70 minutes suggests that apart from antigen internalization, with a half life of 8 minutes, additional factors such as competition for antigen in germinal centers [28,29] can influence the affinity maturation process. The reported biological constants can be used to evaluate the maturation level of antibody repertoires, polyclonal sera and monoclonal antibodies in particular and are hence of value in basic immunological science as well as in antibody drug discovery and vaccine development.

Table 1: Results of Symplex™ repertoire cloning[a].

Donor	*Boost no.*	*Estimated no. of isolated antibody producing cells*[a]	*No. of bacterial clones screened*	*No. of TT-specific clones*	*No. of clones sequenced*	*No. of unique VH-VK amino acid sequences*	*Estimated number of clonotypes*
TT1	V1	400	3600	338	169	120	30[b]
TT1	V2	1700	2100	427	155	108	50
TT1	V3	2400	2600	372	176	154	67
TT2	V1	400	3400	187	102	84	42[b]
TT2	V2	1600	3500	95	82	69	36
TT2	V3	1400	3600	104	83	59	29
Total		**7900**	**18800**	**1523**	**767**	**594**	**195**

[a]Repertoire sizes were estimated by gel electrophoresis of a representative set of single cell RT-PCR samples (from 10-20% of the total). Unique V genes were identified based on the deduced amino acid sequences from DNA sequencing. Antibodies were assigned as the same clonotype if they shared V_H, J_H, V_K, and J_K family segments, heavy chain CDR3 lengths and a consensus sequence in CDR3 for both heavy and light chains. [b]The estimated number of clonotypes from Poulsen et al. [11] (TT1-V1 and TT2-V1) were adjusted from 29 and 40 to 30 and 42, respectively, due to a slightly stricter clonotype definition.

Table 2. Biological constants for affinity maturation at different temperatures[a].

		k_{on}, $M^{-1}\cdot s^{-1}$	k_{off}, s^{-1}	K_D, M [b]	$t_{½}$, min^{-1}, [c]
25℃	**x*, geometric mean**	**2.0×10^{5}**	**7.0×10^{-5}**	**3.4×10^{-10}**	**165**
	68% upper CI (x*·s*)	1.2×10^{6}	2.6×10^{-4}	2.9×10^{-9}	619
	68% lower CI (x*/s*)	3.1×10^{4}	1.9×10^{-5}	3.9×10^{-11}	44
	s*, multiplicative standard deviation [d]	6.4	3.7	8.6	3.7
37℃	**x*, geometric mean**	**1.6×10^{5}**	**1.7×10^{-4}**	**1.0×10^{-9}**	**70**
	68% upper CI (x*·s*)	7.6×10^{5}	5.5×10^{-4}	7.6×10^{-9}	232
	68% lower CI (x*/s*)	3.5×10^{4}	5.0×10^{-5}	1.3×10^{-10}	21
	s*, multiplicative standard deviation [d]	4.6	3.3	7.6	3.3

[a] Values were obtained by fitting the standard equation for a normal distribution to log10-transformed experimentally determined data sets; [b] K_D was calculated as k_{off}/k_{on}; [c] t½ was calculated as ln(2)/(koff/60); [d] Multiplicative standard deviations are unitless;

Materials and Methods

Donors and vaccination

Two healthy donors were boosted with the tetanus vaccine (State Serum Institute, Denmark) containing tetanus toxoid three times each under informed consent. The first and the second repertoires were isolated with a 3.5 years interval and the third repertoires were isolated after another 1.5 years. The project was approved by the regional ethical committee in Copenhagen, Denmark. Blood was harvested with anticoagulant 6 days after vaccination.

Isolation of plasma blasts and cell sorting

Peripheral blood mononuclear cells (PBMC) were purified by density centrifugation using Lymphoprep (Axis-Shield PoC AS) and stained with anti-CD19-FITC (Becton Dickinson) in MACS buffer (phosphate buffered saline (PBS), pH 7.2, 0.5% (w/v) bovine serum albumin (BSA), 2mM EDTA), washed and labeled with anti-FITC conjugated magnetic beads in order to enrich for B cells on a MACS LS column (Miltenyi Biotec) according to the manufacturer's recommendations. The first and the second repertoires were sorted as previously described [11,15]. Shortly, the CD19-enriched fraction was resuspended in FACS buffer (PBS, pH 7.2, 2% fetal calf serum (FCS)) and incubated with anti-CD19-FITC (Becton Dickinson), anti-CD38-APC (Becton Dickinson), anti-lambda-PE (Becton Dickinson), and anti-CD45-PerCP (Becton Dickinson) for 20 minutes at 4°C in the dark, washed and resuspended in FACS buffer and stained for viability with propidium iodide (PI). Viable cells were sorted based on the following expression profile: $CD38^{high}/CD19^{int}/CD45^{int}/lambda^{neg}$. For the first repertoires, single cells were distributed into 96 well microtiter plates by limiting dilution based on manual pipetting. For the second repertoires, single cell sorting was performed on a FACSAria cell-sorting system (Becton Dickinson) where single cells where sorted directly into 384 well PCR plates (Applied Biosystems).

For the third repertoires, the sorting procedure had been optimized to the following: the CD19-enriched B cell fraction was resuspended in FACS buffer (PBS, pH 7.2, 2% FCS) and incubated with anti-CD19-FITC (Becton Dickinson), anti-CD38-PE-Cy7 (Becton Dickinson), anti-CD20-PerCP (Becton Dickinson), anti-CD27-APC (Becton Dickinson),

anti-IgD-PE (Becton Dickinson), and anti-lambda-APC-Cy7 (Becton Dickinson) for 20 minutes at 4°C in the dark, washed and resuspended in FACS buffer. Single cell sorting was performed on a FACSAria cell-sorting system (Becton Dickinson) where plasma blasts were sorted into 384 well PCR plates (Applied Biosystems) based on the following expression profile: $CD19^{int}/CD38^{high}/IgD^{neg}/CD20^{neg}/CD27^{high}/lambda^{neg}$. All FACS analysis was performed using FACSDiva software. 97-99% purity was detected on all single cell sorting.

Plasma blasts were sorted directly into wells containing all reagents necessary for the Symplex PCR except for reverse transcriptase, DNA polymerase and dNTPs as described previously [11] and stored at -80°C for later processing.

Single cell RT-PCR and PCR of antibody variable region genes

V_H and LC_κ genes were amplified and linked through primer overlap extension as described previously [15]. Briefly, V_H and LC_κ from each cell were amplified in a one-step RT-PCR reaction using a cocktail of sense primers specific for the leader regions and antisense primers to the Cγ constant regions for heavy chains and Cκ for the light chain. One microliter from each RT-PCR reaction was transferred to new plates containing reagents for a nested PCR and V_H and LC_κ genes were linked and amplified in separate PCR reactions using nested primers with overlap extensions and containing restriction sites at the ends of the heavy chain variable regions and the full κ light chains as previously described [15].

Cloning and Screening

Linked antibody gene fragments (VH-LCκ) were pooled and cloned into expression vectors using the PCR introduced restriction sites as described previously [11,15]. For the first two repertoires from each donor, linked antibody genes were cloned into a bacterial Fab expression vector [15] and the gene linker was replaced by a bidirectional bacterial promoter fragment (lac-tac) by sub-cloning. To overcome low expression levels for many of the bacterial clones in the first and second repertoires, for the third repertoire from each donor, linked antibody genes were cloned into a mammal Fab expression vector [30] and the gene linker was replaced by a bidirectional mammal promoter fragment (double

CMV promoter) by sub-cloning. The vectors were transformed into bacteria (*E.coli*, TOP10) by electroporation and transformants were selected on 2xYT or LB broth agar containing 100µg/ml of carbenicillin. Single *E.coli* colonies were picked into 96 or 384 well plates. Fabs were expressed directly in *E.coli* by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) which was added to a final concentration of 0.1 mM for screening in the first and second repertoires. Plasmids from clones in the third repertoire were transfected into HEK293 cells in the following manner: 1µl suspension of *E.coli* picked into 384 plates as single clones (CFU) were lysed by incubation for 15 minutes in 4M NaOH. Plasmid DNA was amplified using the TempliPhi Amplification kit (GE Amersham) by transferring 1µl lysed cell suspension to a mix of the kit enzyme and buffer and incubating at 30°C overnight and then inactivating the enzyme for 10 minutes at 65°C. TempliPhi plasmids were transfected into HEK293 cells using Optimem (Invitrogen) and 293Fectin (Invitrogen) in 384 well plates in FreeStyle HEK293 medium (Invitrogen). The plates were incubated shaking at 37°C overnight and Tryptone N1 (Organotechnie) was added to all wells to a final concentration of 0.4%. Plates were incubated while shaking another 72 hours at 37°C.
Both bacterial and mammal clone supernatants were screened for activity against TT; the first repertoires were screened by ELISA and the second and third repertoire from each donor was screened by bead based fluorescence-linked immunosorbent assay (FMAT 8100 HTS; Applied Biosystems). At compatible number of positive clones were selected for DNA sequencing of V genes (Eurofins, MWG/Operon; see table 1). The VH-Vκ sequences were aligned and grouped into clusters according to sequence homology. For each group, the V-D-J usage and location of somatic mutations were determined by alignment with germline sequences using the IMGT sequence directory [16].

Fab production for kinetic measurements

Fab fragments in *E.coli* were produced for SPR analysis (all clones selected for further analysis from the first repertoires and 6 and 4 clones from the second repertoire of donor TT1 and TT2, respectively) essentially as described previously [11]. Due to low bacterial Fab expression levels, it was decided to express Fab in HEK293 during the expression of the second repertoires. The rest of the clones selected for analysis in the second repertoires

were cloned into the mammal HEK293 expression vector, antibody sequences were verified, and plasmids were transfected into HEK293 cells using Optimem (Invitrogen) and 293Fectin (Invitrogen) in FreeStyle HEK293 medium (Invitrogen) in volumes of 30-100 ml. Cultures were incubated while shaking at 37°C for 6 days after which supernatants were harvested by centrifugation. Fab fragments were purified using Poly-prep columns (Bio-Rad) with protein L immobilized on agarose (Pierce) according to the manufacturer's instructions.
The concentration of purified Fab was determined by an indirect immuno assay.

Surface plasmon resonance (SPR)

Affinities (K_D), on-rates (k_a) and off-rates (k_d) were determined by SPR analysis on Biacore 2000 (GE Healthcare/Biacore, Uppsala, Sweden) or Proteon XPR36 (Bio-Rad). Briefly, TT was immobilized at low ligand densities on CM5 chips (Biacore) or GLC chips (Bio-Rad), resulting in maximum response unit (RU) values of ~100 RU or less. Fab fragments were diluted to 96 or 48 nM, and rate constants were measured by injection of at least four serial 2-fold dilutions of the antibodies at 50µl/min. Purified Fab was diluted in running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 on Biacore 2000 and PBS pH 7.2, 0.005% Tween20 on Bio-Rad) and passed over the chip at 50 µl/min.
All measurements were conducted at 25°C or 37°C. Association was measured for 5 min and dissociation for 20-30 min. However, for interactions with very slow off-rates (below $1x10^{-4}$ s^{-1}), dissociation was measured for longer periods of time, up to 16 h, depending on the off-rate. Sensorgrams were fitted globally to a single exponential 1:1 interaction model.using the BIAevalution or Proteon 2.0 software.

Statistics

All statistics were performed using SAS JMP 7.0 or 8.0. Log-transformed experimental data was fitted to the normal distribution:

Normal distribution: $f(x) = \frac{1}{\sqrt{2\pi\sigma}} \exp\left(-\frac{(x-\mu)^2}{2\sigma^2}\right)$

Estimates of the geometric mean and the multiplicative standard deviations describing the normal distribution were obtained using the following expressions:

Geometric mean: $\bar{x}^* = 10\wedge\left(\frac{1}{n}\sum_{i=1}^{n}\left[\log 10\left(x_i\right)\right]\right)$

Multiplicative standard deviation: $s^* = 10\wedge\left(\left[\frac{1}{n-1}\sum_{i=1}^{n}\left[\log 10\left(\frac{x_i}{\bar{x}^*}\right)\right]^2\right]^{1/2}\right)$

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Legends

Figure 1: statistical analysis of the frequency of somatic amino acid mutations in V gene segments and repertoire size. (A-C) The number of somatic mutations for each heavy chains (A), light chains (B) and heavy and light pair combined (C) are plotted for each repertoire (indicated below). Quantile box plots show distributions of the full data set combined containing all six repertoires. The box plots show the maximum, minimum and median values, the 25th and 75th interquartile values, and the 90% and 10% percentiles. The central diamond marks the mean with a 95% confidence interval. To the far right, distributions are shown as histograms including a superimposed fitted normal distribution profiled. "n" indicates the number of unique amino acid sequences in each data set. (D) Analysis of repertoire sizes. The darker shade (bottom column) represents the number of clonotypes observed in at least two of the repertoires from each donor. The lighter shade (top column) represents the clonotypes that are uniquely associated with a single repertoire. Diamonds illustrate statistical maximum likelihood estimates of repertoire sizes derived statistically from the total number of unique sequences and the number of clonotypes from each repertoire [11,20].

Figure 2: limits for affinity maturation measured at 25°C. (a-c) Experimentally determined k_{on} (a), k_{off} (b), and calculated K_D (c) values are plotted for each repertoire as indicated. Medians are shown as black bars. A two-way analysis of variance (ANOVA) was conducted for each data set (on-rates, off-rates, and affinities) to test for differences between repertoires. The arrow indicates that the off-rate of this particular antibody was too slow to measure accurately using SPR. Hence this data point only indicates an upper limit for the off-rate and affinity. (d-f) Statistical analysis of the distribution of binding constants. Distributions of log10 transformed k_{on} (d), k_{off} (e), and calculated K_D (f) plotted as normal quantile plots (upper panels) and histograms (lower panels). Data on all six repertoires were combined in the analysis of on-rate and data on the last two vaccinations were combined for the analysis of the distribution of off-rates and affinities. The log10 transformed data was fitted by the normal distribution to identify the limit for

affinity maturation on kinetic constants. The horizontal line in the normal quantile plots represents the mean while the curved dotted lines are 95% confidence intervals.

Figure 3: Limits for affinity maturation measured at 37°C. (a-c) Binding constants k_{on} (a), k_{off} (b), and calculated K_D (c) were determined from a set of 50 antibodies evenly selected from the four repertoires of the last two booster vaccinations. (d-f) Statistical analysis of the distribution of binding constants measured at physiological temperature as presented in figure 2.

Figure 1

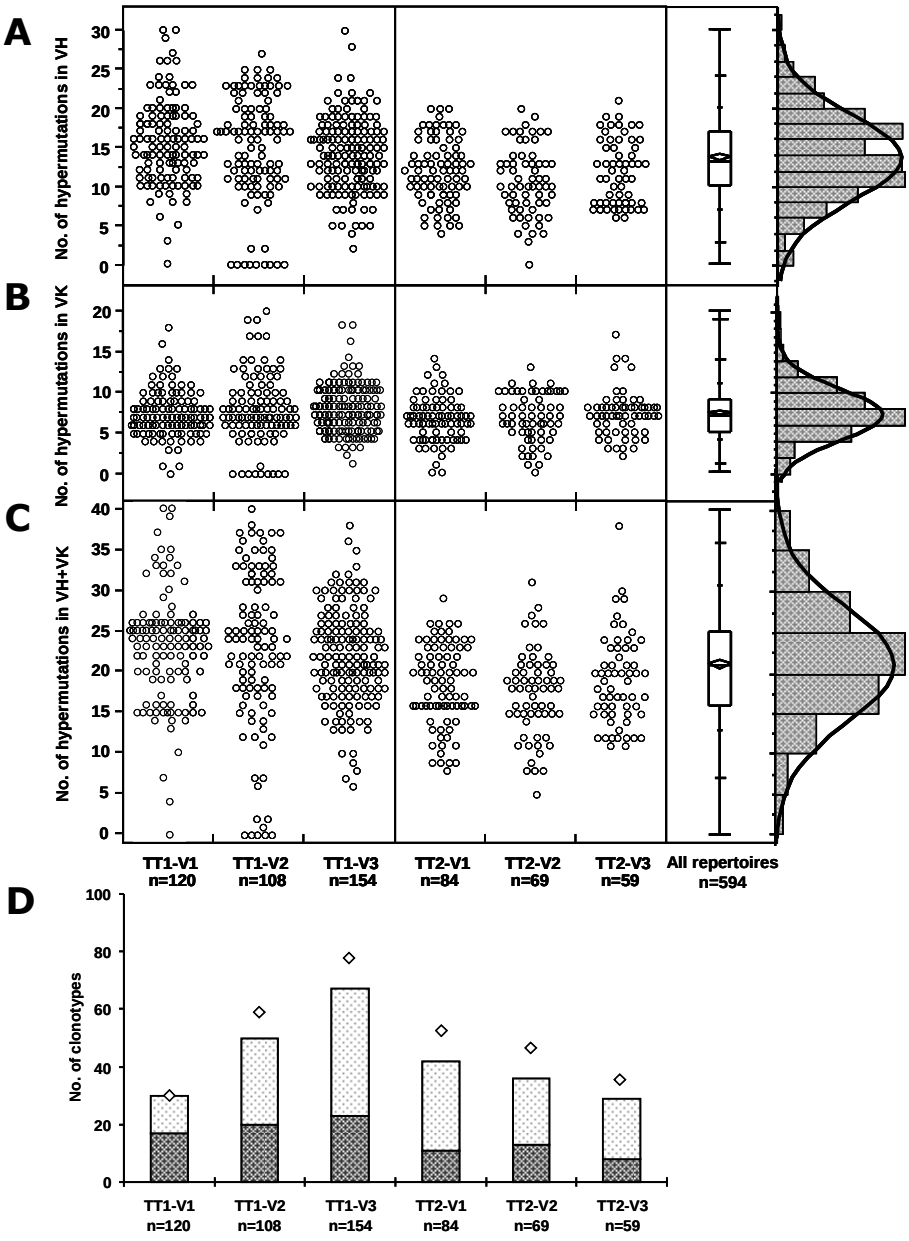

A
No. of hypermutations in VH
B
No. of hypermutations in VK
C
No. of hypermutations in VH+VK
TT1-V1 n=120
TT1-V2 n=108
TT1-V3 n=154
TT2-V1 n=84
TT2-V2 n=69
TT2-V3 n=59
All repertoires n=594
D
No. of clonotypes
TT1-V1 n=120
TT1-V2 n=108
TT1-V3 n=154
TT2-V1 n=84
TT2-V2 n=69
TT2-V3 n=59

Figure 2

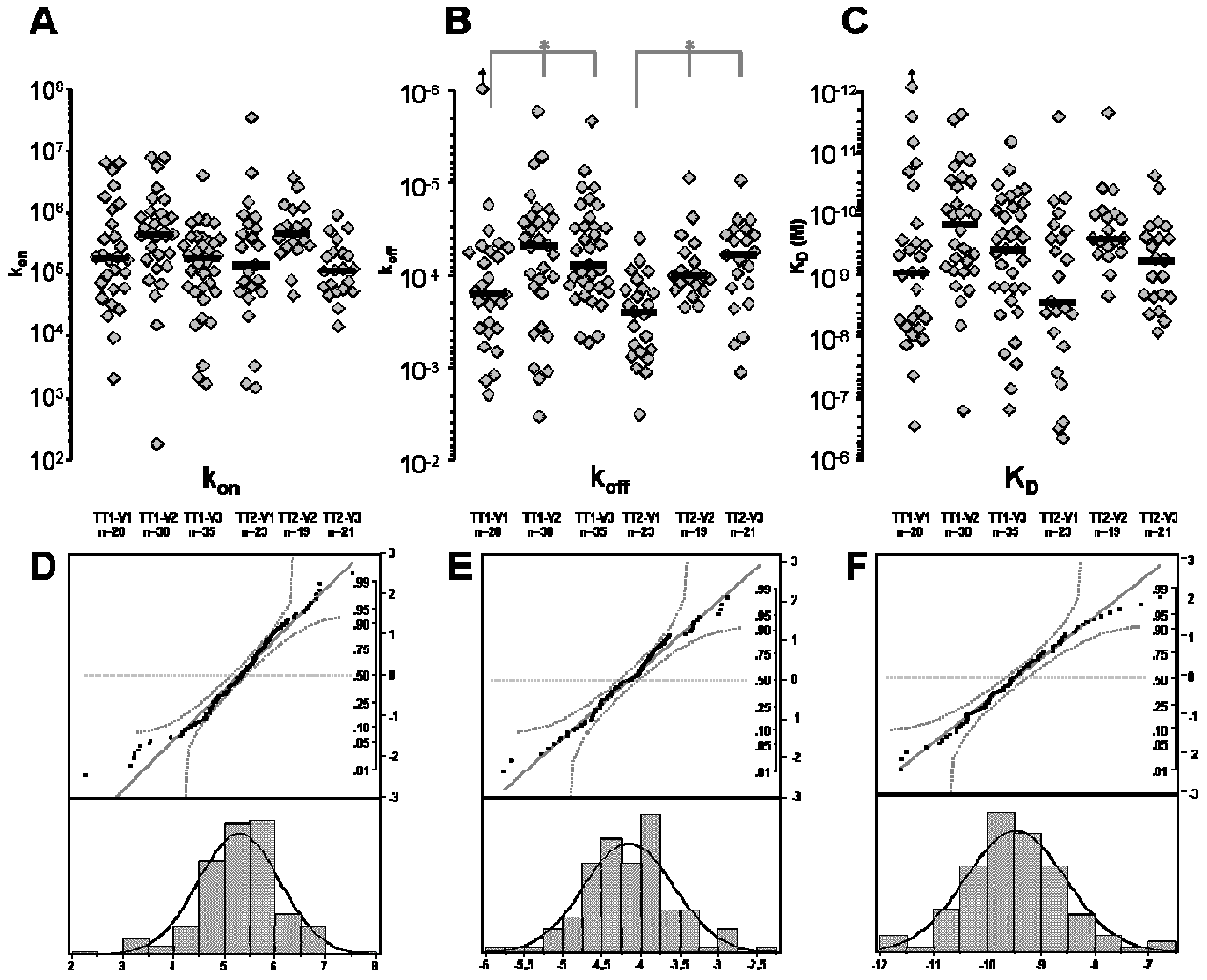
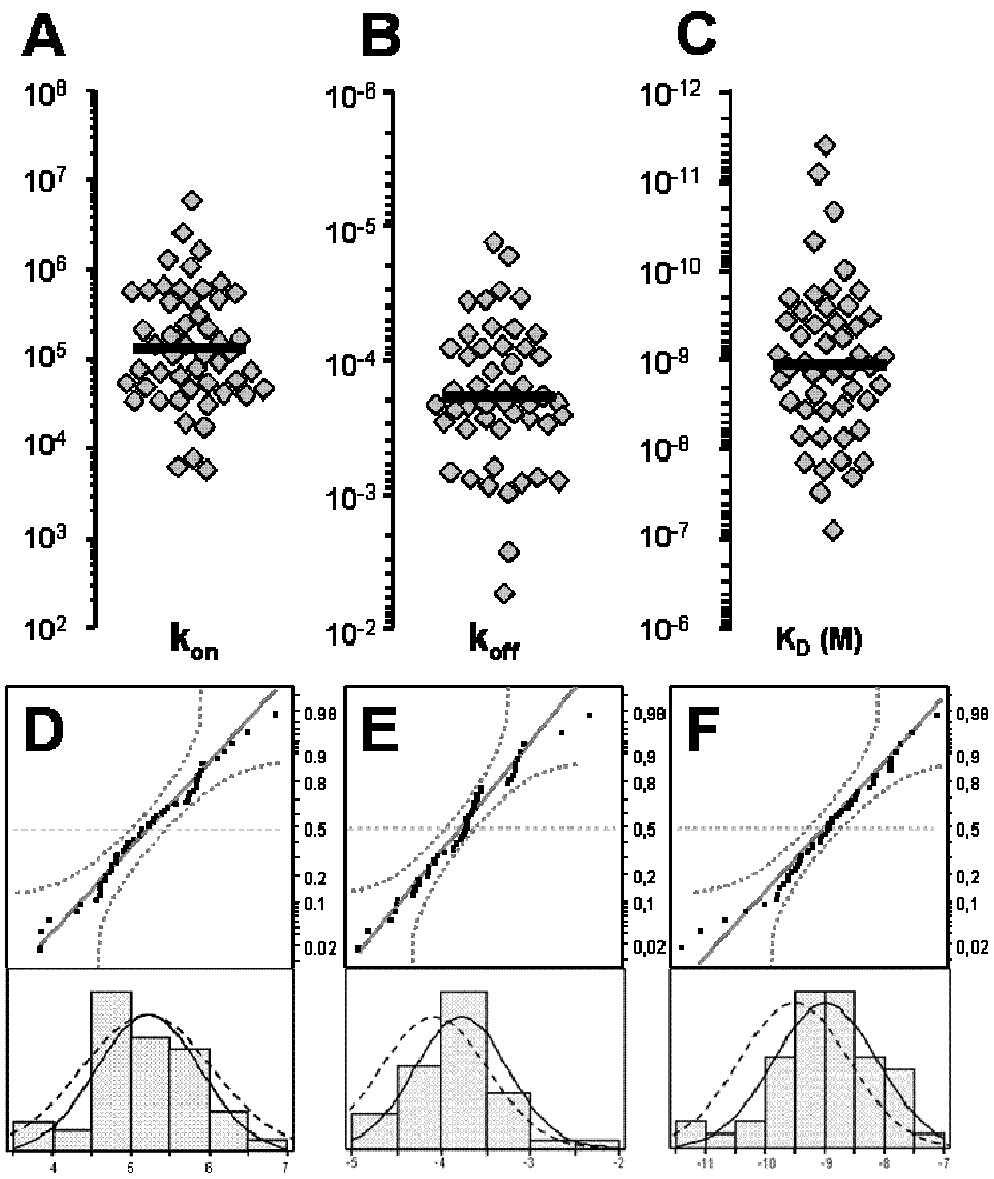

A
B
C
k_{on}
k_{off}
K_D
K_D (M)
TT1-V1 n=20
TT1-V2 n=30
TT1-V3 n=35
TT2-V1 n=23
TT2-V2 n=19
TT2-V3 n=21
D
E
F

Figure 3


A
B
C
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
k_{on}
10^{-8}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
k_{off}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
K_D (M)
D
E
F
0.98
0.9
0.8
0.5
0.2
0.1
0.02
0,98
0,9
0,8
0,5
0,2
0,1
0,02
4
5
6
7
-5
-4
-3
-2
-11
-10
-9
-8
-7

Supplementary information:

Supplementary table 1a. Repertoire and affinities of antibody clonotypes from donor TT1

Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V1	IGHV1-18*01	IGHD1-26*01	IGHJ4*02	CARDFYSGSYRSFDYW	21	IGKV1-39*01	IGKJ1*01	CQQSYSTSWTF	6						
V1	IGHV1-18*01	IGHD1-26*01	IGHJ4*02	CARDFYSGSYRSFDCW	20	IGKV1-39*01	IGKJ1*01	CQQSYSTSWTF	7						
V1	IGHV1-18*01	IGHD1-26*01	IGHJ4*02	CARDFYSGSYRSFDYW	20	IGKV1-39*01	IGKJ1*01	CQQSYSTSWTF	6						
V1	IGHV1-18*01	IGHD4-23*01	IGHJ4*02	CARDFYSGTYRSFDYW	30	IGKV1-39*01	IGKJ1*01	CQQSYGTSWTF	10						
V1	IGHV1-18*01	IGHD4-23*01	IGHJ4*02	CARDFYSGTYRSFDHW	30	IGKV1-39*01	IGKJ1*01	CQQSYGTSWTF	10						
V1	IGHV1-18*01	IGHD4-23*01	IGHJ4*02	CARDFYSGTYRSFDYW	29	IGKV1-39*01	IGKJ1*01	CQQSYGTSWTF	10	2,02E+05	1,97E-03	9,77E-09			
V2	IGHV1-18*01	IGHD4-23*01	IGHJ4*02	CARDFYSGSYRSFDYW	27	IGKV1-39*01	IGKJ1*01	CQQSYGTSWTF	10						
V3	IGHV1-18*01	IGHD4-23*01	IGHJ4*02	CARDFYSGSYRSFDYW	28	IGKV1-39*01	IGKJ1*01	CQQSYGTSWTF	10	1,16E+05	3,07E-05	2,64E-10			
V1	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYHFDYW	13	IGKV1-39*01	IGKJ2*03	CQQSKSPPYNF	10						
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYHFDYW	14	IGKV1-39*01	IGKJ2*03	CQQSKSPPYNF	12						
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYHFDYW	14	IGKV1-39*01	IGKJ2*03	CQQSKSPPYNF	13	1,63E+06	2,30E-05	1,41E-11	2,98E+05	7,23E-05	2,42E-10
V3	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYHFDYW	15	IGKV1-39*01	IGKJ2*03	CQQSKSPPYNF	10						
V3	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CTRDYSSPYYFEFW	17	IGKV1-39*01	IGKJ2*01	CQQSTGPPYTF	7	3,13E+05	2,10E-06	6,71E-12			
V3	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYYFEHW	11	IGKV1-39*01	IGKJ2*01	CQASNGPPYTF	9						
V1	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CVRDYNGSGKYYFEYW	10	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	7						
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CVRDYNGSGKYYFEYW	10	IGKV1-39*01	IGKJ2*01	ND	14						
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSVYYFDYW	22	IGKV1-39*01	IGKJ2*01	CQQSYRTPYTF	12	1,21E+05	4,70E-05	3,90E-10	2,56E+05	6,99E-05	2,73E-10
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSVYYFDYW	22	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	12	4,40E+04	3,30E-05	7,49E-10			
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSVYYFDYW	22	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	13	5,60E+05	1,70E-06	3,03E-12			
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSVYYFDYW	22	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	13						
V2	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSVYYFDYW	23	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	12						
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGSIYYFDYW	17	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	8	1,48E+05	7,50E-06	5,07E-11	8,46E+04	7,28E-05	8,60E-10
V3	IGHV1-18*01	IGHD6-19*01	IGHJ4*02	CARDPRTVAGMSYFLHW	5	IGKV1-39*01	IGKJ2*01	CQQTSSTMYTF	5						
V3	IGHV1-18*01	IGHD6-19*01	IGHJ4*02	CARDPRTVAGMSYFLYW	5	IGKV1-39*01	IGKJ2*01	CQQTSSPMYTF	8						
V1	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	16	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	9	1,40E+06	5,01E-05	3,58E-11			
V1	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	16	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	9						
V1	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	16	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	6	9,62E+04	3,23E-05	3,36E-10			
V1	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	15	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	9						
V3	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	16	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	7	7,39E+05	3,01E-05	4,07E-11	7,18E+06	5,71E-05	7,95E-12
V3	IGHV1-18*01	IGHD3-3*01	IGHJ5*02	CARDRGRITLFGEVILRAGWFDSW	8	IGKV1D-33*01	IGKJ2*02	CQQCDSLPPVF	8						
V2	IGHV1-8*01	ND	IGHJ3*01	CARGGSSAFDVW	19	IGKV1D-33*01	IGKJ3*01	CQVYDNPGATF	13						
V3	IGHV1-3*01	IGHD3-9*01	IGHJ6*02	CTRARNAFYTLPHYDMDVW	15	IGKV2-28*01	IGKJ5*01	CMQPLQAPPTF	3						
V3	IGHV1-3*01	IGHD3-22*01	IGHJ6*02	CTRARNVHYTLPHYDMDVW	18	IGKV2-28*01	IGKJ5*01	CMQPLQAPPTF	6						
V2	IGHV1-3*01	IGHD1-14*01	IGHJ4*02	CARDRWMGGYPRNFFDSW	23	IGKV1-5*03	IGKJ2*02	CQQYNANPATF	17						
V3	IGHV1-69*06	IGHD3-16*01	IGHJ6*03	CARVGGGTRGYYYMAVW	13	IGKV1-27*01	IGKJ3*01	CQKYNDDLFTF	4						
V1	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	15	IGKV1-27*01	IGKJ1*01	CQKYNSDPRTF	4	5,84E+04	6,61E-04	1,13E-08			
V1	IGHV1-69*06	IGHD7-27*01	IGHJ6*03	CARHRGGTLPYYYFDVW	16	IGKV1-27*01	IGKJ1*01	CQKYDSAPRTF	6						
V2	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	15	IGKV1-27*01	IGKJ1*01	CQKYNSEPRTF	6						
V2	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	16	IGKV1-27*01	IGKJ1*01	CQKYNSEPRTF	6	7,79E+06	1,90E-05	2,44E-12			
V3	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	14	IGKV1-27*01	IGKJ1*01	CQKYNSDPRTF	6						
V3	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	14	IGKV1-27*01	IGKJ1*01	CQKYNSDPRTF	5						
V3	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRHYYALDAW	14	IGKV1-27*01	IGKJ1*01	CQKYNSDPRTF	4						
V3	IGHV1-69*06	IGHD4-23*01	IGHJ6*02	CARDYGGTRDYYALDAW	13	IGKV1-27*01	IGKJ1*01	CQKYNSDPRTF	6	3,55E+05	1,35E-04	3,80E-10	1,74E+05	1,84E-04	1,06E-09
V1	IGHV1-69*01	ND	IGHJ6*02	CARVFGGTRLYYALNVW	16	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	6						
V1	IGHV1-69*01	ND	IGHJ6*02	CARVFGGTRLYYALNVW	15	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	8						
V1	IGHV1-69*01	ND	IGHJ6*02	CARVLGGTRLYYALNVW	14	IGKV1-27*01	IGKJ3*01	CQRYDSGLIFTF	8						
V1	IGHV1-69*01	ND	IGHJ6*02	CARVFGGTRLYYALNVW	14	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	6						
V1	IGHV1-69*01	ND	IGHJ6*02	CARVFGGTRLYYALNVW	14	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	6						
V1	IGHV1-69*01	ND	IGHJ6*02	CARVFGGTRLYYALNVW	13	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	6	6,38E+06	1,73E-05	2,71E-12			
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	6						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDGGLRFTF	4						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	6	2,66E+06	5,60E-05	2,11E-11			
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4	4,64E+06	7,35E-05	1,58E-11			
V1	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V2	IGHV1-69*01	ND	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V2	IGHV1-69*01	ND	IGHJ6*02	CARVLGGTRLYYALNVW	11	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V2	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYALNVW	12	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V2	IGHV1-69*01	ND	IGHJ6*02	CGRVLGGTRLYYALNVW	12	IGKV1-27*01	IGKJ3*01	CQKYDSDLIFTF	6	2,14E+05	1,93E-05	9,02E-11			
V2	IGHV1-69*01	ND	IGHJ6*02	CGRVLGGTRLYYALNVW	12	IGKV1-27*01	IGKJ3*01	CQKYDSGLIFTF	7						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYALNVW	9	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYALNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V3	IGHV1-69*01	IGHD6-19*01	IGHJ6*02	CARVLGGTRVYYALNVW	13	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	5						
V3	IGHV1-69*01	IGHD3-16*02	IGHJ6*02	CARVFGGTRLYYALNVW	13	IGKV1-27*01	IGKJ3*01	CQKYDSGLKFTF	7						
V3	IGHV1-69*01	IGHD4-17*01	IGHJ6*02	CARVLGGTRLYYALNVW	15	IGKV1-27*01	IGKJ3*01	CQKYDSALIFTF	5						
V3	IGHV1-69*01	IGHD2-2*03	IGHJ6*02	CARVLGGTRLYYALHIW	19	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	6						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYAQNVW	10	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYAQNVW	9	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTF	4						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYAQNVW	9	IGKV1-27*01	IGKJ3*01	CQKYDSGLRFTL	4						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYAMNVW	9	IGKV1-27*01	IGKJ3*01	CQKYDSGLKFTF	5	7,60E+05	5,10E-05	6,71E-11			
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARVLGGTRLYYAMNVW	9	IGKV1-27*01	IGKJ3*01	CQKYDSGLKFTF	5						
V3	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	CARLLGGTRHYYALNVW	20	IGKV1-27*01	IGKJ3*01	CQKYDSALIFTF	6						
V1	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGFW	11	IGKV1-27*01	IGKJ3*01	CQEYSNALIFTF	5						
V2	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGFW	12	IGKV1-27*01	IGKJ3*01	CQEYSNALIFTF	6						
V2	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGFW	13	IGKV1-27*01	IGKJ3*01	CQEYSNALIFTF	6	6,82E+04	5,95E-05	8,72E-10	4,11E+04	4,99E-05	1,22E-09
V3	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGVW	13	IGKV1-27*01	IGKJ3*01	CQEYSSALIFTF	7						
V3	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGFW	10	IGKV1-27*01	IGKJ3*01	CQEYSNALIFTF	6	3,99E+06	7,72E-05	1,94E-11			
V3	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRPYYALGLW	10	IGKV1-27*01	IGKJ3*01	CQEYSNVLIFTF	6						
V3	IGHV1-69*06	IGHD1-26*01	IGHJ6*02	CARVVGGTRSYYALGVW	16	IGKV1-27*01	IGKJ3*01	CQEYSSALIFTF	7						
V1	IGHV1-69*06	ND	IGHJ6*03	CARDRGGTRHHYYMDVW	24	IGKV3-20*01	IGKJ2*02	CQQYSGPLRTF	8	6,37E+06	4,55E-05	7,15E-12			
V1	IGHV1-69*06	ND	IGHJ6*03	CARDRGGTRHHYYMDVW	22	IGKV3-20*01	IGKJ2*02	CQQYSGPLRTF	8						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDVW	14	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	12						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDVW	14	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	12						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDVW	14	IGKV3-20*01	IGKJ1*01	CQ.YGDSVFTF	11						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDVW	14	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	11						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDIW	14	IGKV3-20*01	IGKJ1*01	CQRYGDSVFTF	11						
V1	IGHV3-21*01	ND	IGHJ6*03	CTRCRGGTRTYYYMDVW	15	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	11	1,18E+05	6,33E-05	5,36E-10			
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CTRCRGGTRTYYYMDVW	15	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	11						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRSYYYMDVW	10	IGKV3-20*01	IGKJ1*01	CQQYGDSVFTF	10						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRSYYYMDVW	19	IGKV3-20*01	IGKJ1*01	CQQYGDSLNTF	10						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRHYYYLDVW	9	IGKV3-20*01	IGKJ1*01	CQQYVSSFSTF	8						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRHYYYLDVW	9	IGKV3-20*01	IGKJ1*01	CQQYVSSFSTF	7						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRQYYYLDVW	9	IGKV3-20*01	IGKJ1*01	SQQYVSSFSTF	11						
V3	IGHV3-21*01	IGHD2-15*01	IGHJ6*03	CARCRGGTRQYYYLDVW	9	IGKV3-20*01	IGKJ1*01	CQQYVSSFSTF	9						

Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V2	IGHV1-69*06	IGHD2-2*02	IGHJ4*02	CASGYCSSTSCYDYW	0	IGKV3-20*01	IGKJ5*01	CQQYGSSPYSF	6						
V2	IGHV1-69*01	IGHD3-22*01	IGHJ5*02	CARRHGYSDQRNWFDSW	17	IGKV1-39*01	IGKJ2*01	CQQCYETPYTF	8						
V2	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDYW	16	IGKV1-5*03	IGKJ2*02	CQQYNSLQGTF	6	**5,35E+06**	**1,57E-04**	**2,93E-11**			
V2	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDYW	16	IGKV1-5*03	IGKJ2*02	CQQYNSLQGTF	7						
V2	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDYW	17	IGKV1-5*03	IGKJ2*02	CQQYNSLQGTF	6	**1,57E+06**	**1,11E-04**	**7,07E-11**			
V2	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDFW	17	IGKV1-5*03	IGKJ2*02	CQHYNSLRGTF	11	**7,91E+04**	**2,64E-05**	**3,34E-10**			
V3	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDFW	17	IGKV1-5*03	IGKJ2*02	CQHYNSLRGTF	12						
V3	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGSNFVYFDFW	16	IGKV1-5*03	IGKJ2*02	CQHYNSLRGTF	10						
V3	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGNNFVYFDYW	16	IGKV1-5*03	IGKJ2*02	CQQYNSLQGTF	10	**1,12E+05**	**1,05E-04**	**9,38E-10**			
V3	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	CALRDGNNLVYFDYW	15	IGKV1-5*03	IGKJ2*02	CQHYNSLQGTF	10						
V2	IGHV1-69*01	IGHD1-26*01	IGHJ4*01	CARLYSGSYYPVEYW	18	IGKV3-20*01	IGKJ1*01	CHQYGSSPRTF	19						
V2	IGHV1-69*01	IGHD1-26*01	IGHJ4*01	CARLYSGSYYPVEYW	17	IGKV3-20*01	IGKJ1*01	CHQYGSSPRTF	19						
V2	IGHV1-69*01	IGHD1-26*01	IGHJ4*01	CARLYSGSYYPVEYW	17	IGKV3-20*01	IGKJ1*01	CHQYGSSPRTF	20						
V2	IGHV1-69*11	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	23	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	10						
V2	IGHV1-69*06	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	23	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	10	**4,18E+05**	**3,69E-04**	**8,83E-10**			
V2	IGHV1-69*06	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	23	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	10						
V2	IGHV1-69*06	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	21	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	7						
V2	IGHV1-69*11	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	19	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	8	**2,45E+06**	**4,64E-04**	**1,90E-10**			
V2	IGHV1-69*06	IGHD6-13*01	IGHJ4*02	CARVREGQQVLVFDSW	19	IGKV1-5*03	IGKJ1*01	CQHYNSLRGTF	9						
V2	IGHV1-24*01	ND	IGHJ4*02	CATGRGLEFDYW	16	IGKV1-5*03	IGKJ1*01	CQQFHTYRTF	6						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFNGYNLGGYMDVW	15	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	7						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFNGYNLGGYMDVW	14	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	6						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFNGYNLGGYMDVW	13	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	6	**2,66E+04**	**1,20E-03**	**4,50E-08**			
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDVW	11	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	5						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDVW	10	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	5	**2,09E+04**	**1,52E-04**	**7,28E-09**			
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDVW	10	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	6						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDVW	10	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	5						
V1	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDVW	10	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF	5						
V2	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDLW	8	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	4						
V2	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFTGYNLGGYMDLW	9	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	4	**7,06E+05**	**1,29E-03**	**1,83E-09**			
V2	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSHDVFTGYNLGGYMDLW	9	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	6	**3,95E+05**	**1,09E-03**	**2,77E-09**			
V3	IGHV5-51*01	IGHD3-9*01	IGHJ6*03	CARHLDSYDVFDGYNLGGYMDVW	14	IGKV3-20*01	IGKJ1*01	CQQYGSSPQTF	8						
V2	IGHV5-51*01	IGHD2-15*01	IGHJ4*02	CARHRGGWAKRGPFDYW	18	IGKV1-5*03	IGKJ2*01	CQQYHDYSPEYTF	7						
V2	IGHV5-51*01	IGHD2-15*01	IGHJ4*02	CARHRGGWAKRGPFDYW	17	IGKV1-5*03	IGKJ2*01	CQQYHDYSPEYTF	7						
V2	IGHV5-51*01	IGHD2-15*01	IGHJ4*02	CARHRGGWAKRGPFDYW	17	IGKV1-5*03	IGKJ2*01	CQQYHDYSPEYTF	7						
V3	IGHV5-51*01	IGHD2-15*01	IGHJ4*02	CARHRGGWAKRGPFDYW	15	IGKV1-5*03	IGKJ2*01	CQQYHSYSPEYTF	7						
V3	IGHV5-51*01	ND	IGHJ4*02	CARHRGGWAKRGPFDYW	13	IGKV1-5*03	IGKJ2*01	CQQYHSYSPEYTF	7						
V1	IGHV5-51*01	IGHD3-10*02	IGHJ4*02	CARQDSNTLYFFDLW	11	IGKV3-11*01	IGKJ2*01	CQHRSKYTF	7						
V1	IGHV3-21*01	IGHD2-15*01	IGHJ3*02	CARRPGWAATRAAGAFDIW	0	IGKV1-27*01	IGKJ2*01	CQKYNSAPYTF	0	**9,10E+03**	**1,29E-04**	**1,42E-08**			
V1	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	14	IGKV1-39*01	IGKJ1*01	CQQIYSQVRTF	10						
V1	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	14	IGKV1-39*01	IGKJ1*01	CQQIYSQVRTF	9						
V1	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	13	IGKV1-39*01	IGKJ1*01	CQQIYSHVRTF	9						
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	13	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	5	**2,02E+05**	**9,61E-05**	**4,75E-10**			
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	12	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	5						
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	11	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	5						
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	11	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	6						
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CQQIYSHVRTF	12	**4,89E+05**	**3,42E-03**	**6,98E-09**	**2,36E+04**	**3,25E-05**	**1,38E-09**
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CQQIYSHVRTF	11						
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CPQIYSNVRKF	5	**9,58E+05**	**1,05E-04**	**1,10E-10**			
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	4						
V3	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CQQIYSHVRTF	9	**1,01E+05**	**1,66E-04**	**1,64E-09**	**7,22E+05**	**2,63E-04**	**3,64E-10**
V3	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	12	IGKV1-39*01	IGKJ1*01	CQQIYSNVRTF	5						
V3	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CQQIYSHVRTF	11	**4,00E+05**	**9,82E-05**	**2,46E-10**	**1,93E+05**	**3,01E-05**	**1,56E-10**
V3	IGHV3-21*01	ND	IGHJ4*02	CASGNTHDYW	10	IGKV1-39*01	IGKJ1*01	CLQTYSNVRTF	7						
V3	IGHV3-21*01	ND	IGHJ4*02	CLTGSSLDYW	7	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTF	10						
V3	IGHV3-21*01	ND	IGHJ4*02	CASGVTHDYW	19	IGKV1-9*01	IGKJ1*01	CLQRYTYRRTF	10						
V3	IGHV3-21*01	ND	IGHJ4*02	CASGRSLDYW	10	IGKV1-39*01	IGKJ1*01	CHQTYCTTRTF	6	**3,94E+04**	**1,80E-04**	**4,57E-09**			
V2	IGHV3-21*01	ND	IGHJ4*02	CASGNTLDYW	17	IGKV3-20*01	IGKJ2*02	CHQYCYSRTF	8						
V3	IGHV3-21*01	ND	IGHJ4*02	CATGRTLDYW	10	IGKV3-20*01	IGKJ1*01	CHQYPTSRAF	11						
V1	IGHV3-21*01	ND	IGHJ4*02	CVSGSSFDYW	16	IGKV1-39*01	IGKJ1*01	CLQTYSSHRTF	10						
V1	IGHV3-21*01	ND	IGHJ4*03	CVTGSSLDYW	15	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTF	14						
V1	IGHV3-21*01	ND	IGHJ4*02	CVTGSSLDYW	14	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTF	13	**1,04E+05**	**4,19E-04**	**4,04E-09**			
V3	IGHV3-21*01	ND	IGHJ4*02	CVTGSSLDYW	12	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTF	11						
V3	IGHV3-21*01	ND	IGHJ4*02	CVTGSSLDYW	11	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTL	12						
V3	IGHV3-21*01	ND	IGHJ4*02	CVTGSSLDYW	7	IGKV1-39*01	IGKJ1*01	CLQTYSVIRTF	10	**5,06E+04**	**1,33E-04**	**2,63E-09**	**2,11E+04**	**1,51E-04**	**7,18E-09**
V3	IGHV3-21*01	ND	IGHJ4*02	CVTGSSLDYW	7	IGKV1-39*01	IGKJ1*01	CLQTYCVIRTF	11						
V1	IGHV3-23*01	IGHD4-17*01	IGHJ4*02	CAKDLYGDYDLDYW	6	IGKV2-30*01	IGKJ4*01	CMQGTHWPPITF	1						
V1	IGHV3-23*01	IGHD6-19*01	IGHJ4*02	CAKAHKQWLAHYNFDYW	8	IGKV3-20*01	IGKJ4*01	CQQHDTSPLTF	5						
V2	IGHV3-20*01	IGHD1-26*01	IGHJ3*02	CAGAKVGVGGENAFDIW	10	IGKV4-1*01	IGKJ2*01	CQQYYSTQYTF	9						
V1	IGHV3-64*05	IGHD6-19*01	IGHJ4*02	CVKRRRQWLVNSSFDFW	17	IGKV3-15*01	IGKJ5*01	CQQYHVWPPITF	18						
V1	IGHV3-64*05	IGHD6-19*01	IGHJ4*02	CVKRRRQWLVNSSFDFW	20	IGKV1-5*03	IGKJ1*01	CQQYNPYSWTF	6	**5,77E+04**	**3,22E-04**	**5,57E-09**			
V3	IGHV3-64*05	IGHD6-19*01	IGHJ4*02	CVKRRRQWLVNSSFDFW	17	IGKV1-5*03	IGKJ1*01	CQQYNPYSWTF	4						
V3	IGHV3-64*05	IGHD6-19*01	IGHJ4*02	CVKRRRQWLVNSSFDFW	18	IGKV1-5*03	IGKJ1*01	CQQYNPYSWTF	4						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V1	IGHV3-30-3*01	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V1	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V2	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5	**2,74E+05**	**4,10E-05**	**1,03E-10**	**5,27E+05**	**9,34E-05**	**1,77E-10**
V2	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	21	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5						
V2	IGHV3-30*14	IGHD3-3*01	IGHJ4*02	CARDRGAHLTLFGEPLIRPSSFDFW	25	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V2	IGHV3-30*14	IGHD3-3*01	IGHJ4*02	CARDRGAHLTLFGEPLIRPSSFDFW	25	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V2	IGHV3-30*14	IGHD3-3*01	IGHJ4*02	CARDRGAHLTLFGEPLIRPSSFDFW	25	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	8	**8,04E+05**	**2,47E-05**	**3,07E-11**	**3,78E+05**	**1,93E-04**	**5,12E-10**
V3	IGHV3-30*03	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSR	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6	**1,76E+05**	**3,68E-05**	**2,10E-10**	**2,19E+05**	**8,40E-05**	**3,84E-10**
V3	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	19	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	5	**2,32E+05**	**1,74E-05**	**8,62E-11**	**4,15E+04**	**8,44E-05**	**2,04E-09**
V3	IGHV3-30*04	IGHD3-3*01	IGHJ4*02	CARDRGAQITLFGAPLIRPSSFDSW	20	IGKV1D-33*01	IGKJ4*01	CQQYDGPVLTF	6						
V1	IGHV3-30*04	IGHD2-15*01	IGHJ4*02	CARARLLFCSGGRCDMDSW	16	IGKV1-9*01	IGKJ1*01	CQQIIRYPRTF	7	**1,78E+06**	**1,53E-07**	**8,60E-14**			
V1	IGHV3-30*04	IGHD2-15*01	IGHJ4*02	CARARLLFCSGGRCDMDSW	16	IGKV1-9*01	IGKJ1*01	CQQIIRYPRTF	7						
V2	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	14	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	7	**1,67E+05**	**6,32E-06**	**3,79E-11**	**5,84E+05**	**2,70E-05**	**4,62E-11**
V2	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	15	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	7						
V2	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	17	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	7						
V3	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	12	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	5						
V3	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	13	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	5	**2,84E+05**	**3,03E-05**	**1,07E-10**			
V3	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	13	IGKV1-9*01	IGKJ1*01	CQHLIRYPRTF	5						
V3	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	13	IGKV1-9*01	IGKJ1*01	CQQPIRYPRTF	6	**3,34E+05**	**1,13E-05**	**3,39E-11**	**3,14E+06**	**1,19E-05**	**3,78E-12**
V3	IGHV3-30*03	IGHD2-15*01	IGHJ4*02	CARARLLYCSGGRCDMDYW	14	IGKV1-9*01	IGKJ1*01	CQQLIRYPRTF	8						

Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V2	IGHV3-30*18	IGHD3-22*01	IGHJ4*02	CAKEPAPFNYYDSSAYYGGGYYFDYW	9	IGKV1-5*03	IGKJ2*01	CHQYGSSFPYSF	9						
V2	IGHV3-30*18	IGHD3-22*01	IGHJ4*02	CAKEPAPFNYYDSSAYYGGGYYFDYW	9	IGKV1-5*03	IGKJ2*01	CHQYGSSFPYSF	9						
V2	IGHV3-30*18	IGHD3-16*02	IGHJ3*02	CAKDRQLKDAFDIW	0	IGKV1D-33*01	IGKJ3*01	CQQYDNLPPL	1	1,01E+06	2,63E-05	2,6E-11			
V2	IGHV3-30*18	IGHD3-22*01	IGHJ5*02	CAKGLSQALNYYGSGSPFL	11	IGKV3-20*01	IGKJ1*01	CQQYGTSPWTF	14						
V2	IGHV3-33*03	IGHD2-15*01	IGHJ5*02	CARDICSAGNCYPGNGLDPW	11	IGKV3-20*01	IGKJ1*01	CQHYGSSSWTF	10						
V2	IGHV3-30*18	IGHD2-8*02	IGHJ4*02	CAKDLESFYCTDGGCPFDYW	18	IGKV1-5*03	IGKJ1*01	CQHYASHSQTF	9						
V2	IGHV3-30*18	IGHD2-8*02	IGHJ4*02	CAKDLESFYCTDGGRPFDYW	18	IGKV1-5*03	IGKJ1*01	CQHYASHSQTF	9						
V2	IGHV3-30*18	IGHD2-8*02	IGHJ4*02	CAKDLESFYCTDGGCPFDYW	17	IGKV1-5*03	IGKJ1*01	CQHCASHSQTF	8						
V2	IGHV3-30*18	IGHD3-9*01	IGHJ4*02	CTKDRYRGHVLTGNSFEHW	13	IGKV1-39*01	IGKJ1*01	CQQSYHARWTF	8						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNVRVNLGRGVFDFW	17	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	7						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNVRVNLGRGVFDFW	17	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	7						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNVRVNLGRGVFDFW	18	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	7						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNVRVNLGRGVFDFW	18	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	6						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNVRVNLGRGVFDFW	17	IGKV1D-16*01	IGKJ4*01	CQQYYSSPLSF	6						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNLRVNLGRGVFDFW	13	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	8	7,66E+06	1,30E-04	1,70E-11			
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNLRVNLGRGVFDFW	13	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	9						
V2	IGHV3-30-3*01	IGHD3-10*01	IGHJ4*02	CARGGPGNLRVNLGRGVFDFW	13	IGKV1D-16*01	IGKJ4*01	CQQYYSFPLSF	9						
V2	IGHV3-64*05	IGHD3-22*01	IGHJ3*02	CVKDPRRRTWFFHRSGFDIW	24	IGKV1-9*01	IGKJ5*01	CQQFNSYPITF	14	1,31E+05	8,20E-05	6,26E-10			
V2	IGHV3-64*05	IGHD3-22*01	IGHJ3*02	CVKDPRRRTWFFHRSGFDIW	23	IGKV1-9*01	IGKJ5*01	CQQFNSYPITF	14						
V2	IGHV3-64*05	IGHD3-22*01	IGHJ3*02	CVKDPRRRTWFFHRSGFDIW	23	IGKV1-9*01	IGKJ5*01	CQQFNSYPITF	13	4,36E+05	4,86E-05	1,11E-10	7,52E+05	1,86E-04	2,47E-10
V1	IGHV3-15*01	IGHD4-23*01	IGHJ4*02	CTTHDYW	5	IGKV1-9*01	IGKJ4*01	CQQLDTYPLTL	5						
V2	IGHV3-15*01	IGHD3-10*01	IGHJ4*02	CTTGTVVVPNDYW	0	IGKV3-20*01	IGKJ4*01	CQQYGSSPKLTF	7						
V2	IGHV3-15*01	IGHD3-3*01	IGHJ6*02	CTTDFSILRAYLLRFLEWPSYGMDVW	2	IGKV1-16*01	IGKJ5*01	CQQYNSYCHL	0						
V2	IGHV3-15*01	ND	IGHJ4*02	CATDGAGYSSNLW	11	IGKV1-39*01	IGKJ2*02	CQQSYGTPLTF	0						
V2	IGHV3-15*01	ND	IGHJ4*03	CATDGAGYSSNLW	12	IGKV1-39*01	IGKJ2*02	CQQSYSTPLTF	0						
V2	IGHV3-15*07	IGHD1-26*01	IGHJ1*01	CATGGDIGSYHSGGYFHHW	7	IGKV2-28*01	IGKJ4*01	CMQALQIPLTF	7						
V2	IGHV3-49*03	IGHD2-2*02	IGHJ6*02	CSPRGDCSSTNCYENFFHDLDVW	18	IGKV1D-33*01	IGKJ3*01	CQQYDTLFSF	17						
V2	IGHV3-49*03	IGHD2-2*02	IGHJ6*02	CSPRGDCSSTNCYENFFHDLDVW	20	IGKV1D-33*01	IGKJ3*01	CQQYDTLFSF	17						
V1	IGHV4-39*01	IGHD1-1*01	IGHJ4*02	CARIVGYNWKGEGNFDYW	26	IGKV1-39*01	IGKJ4*01	CQQSFSTPQTF	7	4,03E+05	1,95E-04	4,84E-10			
V3	IGHV4-39*01	IGHD6-19*01	IGHJ4*02	CARQEPKQWLVDYYFDSW	18	IGKV3-20*01	IGKJ1*01	CLQYGSSPWTF	18						
V3	IGHV4-39*01	IGHD6-6*01	IGHJ4*02	CARLQSFYTSQNSVRRPFDSW	17	IGKV1-5*03	IGKJ2*01	CQQYTTYLYTF	5						
V3	IGHV4-39*01	IGHD3-16*01	IGHJ4*02	CARLQSFYTSQNSVRRPFDSW	17	IGKV1-5*03	IGKJ2*01	CQQYTTYLYTF	5						
V1	IGHV4-59*02	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	12	IGKV1-39*01	IGKJ1*01	CQQSYDMPRTF	8						
V1	IGHV4-59*02	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	12	IGKV1-39*01	IGKJ1*01	CQQSYDMPRTF	8	1,69E+05	4,70E-05	2,78E-10			
V1	IGHV4-59*01	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	12	IGKV1-39*01	IGKJ1*01	CQQSYSMPRTF	8						
V1	IGHV4-59*02	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	12	IGKV1-39*01	IGKJ1*01	CXQSXDMPRTF	16						
V1	IGHV4-59*02	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	12	IGKV1-39*01	IGKJ1*01	CQQSYDMPRTF	9						
V1	IGHV4-59*01	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDYW	11	IGKV1-39*01	IGKJ1*01	CQQSYSMPRTF	8	3,14E+04	5,47E-05	1,74E-09			
V1	IGHV4-59*01	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDFW	11	IGKV1-39*01	IGKJ1*01	CQQSYSMPRTF	10						
V1	IGHV4-59*01	IGHD6-25*01	IGHJ4*02	CARVSGWGPRGGIYFDFW	10	IGKV1-39*01	IGKJ1*01	CQQSYSMPRTF	13						
V3	IGHV4-59*01	IGHD5-5*01	IGHJ4*02	CARGGRYSYGYASFFDYW	14	IGKV1-39*01	IGKJ4*01	CQQSYSLPFTF	11						
V3	IGHV4-59*01	IGHD5-5*01	IGHJ4*02	CARGGRYSYGYASFFDYW	14	IGKV1-39*01	IGKJ4*01	CQQSYSLPFTF	13						
V2	IGHV4-59*01	IGHD3-16*02	IGHJ4*02	CVRPPSWRYHYFDSW	20	IGKV3-15*01	IGKJ4*02	CQQYDKWPPEITF	11						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFGSW	19	IGKV3-15*01	IGKJ4*01	CQQYDKWPPEPTF	11						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFGSW	19	IGKV3-15*01	IGKJ4*01	CQQYDKWPPEPTF	11						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	17	IGKV3-15*01	IGKJ4*01	CQQYDKWPPELTF	16						
V3	IGHV4-59*01	IGHD3-16*02	IGHJ4*02	CARPPSWRYHYFDSW	17	IGKV3-15*01	IGKJ4*01	CQQYDKWPPEPTF	10						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	16	IGKV3-15*01	IGKJ4*01	CQQYDKWPPEITF	8						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	16	IGKV3-15*01	IGKJ4*01	CQQYDKWPPELTF	8						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	16	IGKV3-15*01	IGKJ4*02	CQQYDRWPPELTF	8						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	15	IGKV3-15*01	IGKJ4*01	CQQYDKWPPELTF	8						
V3	IGHV4-59*01	IGHD6-13*01	IGHJ4*02	CARPPSWRYHYFDSW	14	IGKV3-15*01	IGKJ4*01	CQQYDKWPPELTF	8						
V2	IGHV4-34*01	IGHD5-12*01	IGHJ6*02	CARLPIIGSGYDAVSLGNYGMDVW	0	IGKV5-2*01	IGKJ4*01	CLQHDNF	0						
V2	IGHV4-34*01	IGHD1-7*01	IGHJ3*02	CARVPLFFSNWNLRAFDIW	2	IGKV1D-33*01	IGKJ4*01	CQQYDNLPLTF	0						
V3	IGHV4-34*01	IGHD4-17*01	IGHJ5*02	CSRVGPFDPW	11	IGKV1-39*01	IGKJ1*01	CQQIYNTPYTF	9	2,81E+05	1,27E-04	4,52E-10	2,03E+05	2,46E-04	1,21E-09
V3	IGHV4-34*01	IGHD4-17*01	IGHJ5*02	CSRVGPFDPW	11	IGKV1-39*01	IGKJ2*01	CQQIYNTPYTF	10						
V3	IGHV4-34*01	IGHD4-17*01	IGHJ5*02	CSRVGPFDPW	11	IGKV1-39*01	IGKJ2*01	CQQIYNTPYTF	12						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVTAPDDTGTILARHNRYYFDSW	17	IGKV3-20*01	IGKJ2*01	CHQYGSSPNTF	8						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVTAPDDTGTILARHNRYYFDSW	18	IGKV3-20*01	IGKJ2*01	CHQYGSSPNTF	8						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	22	IGKV3-11*01	IGKJ2*01	CQQRRLWYTF	3						
V1	IGHV1-69*10	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	23	IGKV3-11*01	IGKJ2*01	CQQRRLWYTF	3						
V1	IGHV1-69*10	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	23	IGKV3-11*01	IGKJ2*01	CQQRRLWYTF	4						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	18	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	8						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	18	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	7						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	19	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	7						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	18	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	7						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	18	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	7						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	19	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	7	1,85E+05	1,70E-04	9,18E-10			
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	19	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	7	6,49E+05	1,98E-04	3,05E-10			
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	10						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	23	IGKV1-5*03	IGKJ2*02	CHQYHSYSGTF	9						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	24	IGKV1-5*03	IGKJ2*02	CHQYHSYSGTF	13						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	26	IGKV3-20*01	IGKJ2*01	CHQYGSTDNTF	8						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	26	IGKV1-39*01	IGKJ2*02	CQQSYRTPCTF	7						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	27	IGKV1-39*01	IGKJ2*01	CQQSYRTPYTF	8						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDTSTIAARFNRYFFDFW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	11						
V1	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDTSTIAARFNRYFFDFW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8	3,68E+05	3,76E-04	1,02E-09			
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDTSTIAARLNRYFFDFW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8	6,40E+05	1,02E-03	1,59E-09	5,06E+04	1,40E-04	2,77E-09
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDASTIAARFNRYFFDFW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8						
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDTSTIAARFNRYFFDFW	24	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8						
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRDTSTIAARFNRYFFDFW	24	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	9	8,00E+05	4,32E-04	5,4E-10			
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRDTSTIAARFNRYFFDSW	24	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8						
V2	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVRAPRGTSTIAARFNRYFFDSW	23	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTISARFNRYFFESW	18	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	7	1,50E+05	8,67E-05	5,78E-10	6,54E+04	2,44E-04	3,74E-09
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CATAPRGTSTIAARFNRYFFDSW	18	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	7	6,72E+05	4,58E-04	6,82E-10	1,46E+05	2,39E-03	1,64E-08
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	18	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	7						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	18	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	6						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVSAPRDTSTIAARFNRYFFDTW	19	IGKV3-20*01	IGKJ2*01	CHQYGASDNTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRGTSTIAARHNRYFFDYW	19	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ6*01	CASAPRGTSTIAARFNRYFFDFW	19	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	9						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CASAPRGTSTIAARFNRYFFDFW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	10						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRDTSTIAARHNRYQFDSW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	10	4,07E+05	1,53E-04	3,76E-10	4,87E+04	6,73E-04	1,38E-08
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPPGTSTIAARFNRYFFDSW	22	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	9						
V3	IGHV1-69*10	IGHD6-6*01	IGHJ4*02	CERAPPGTSTIAARFNRYFFDSW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSENTF	9						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPPGTSTIAARFNRYFFDSW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	10						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRGTSTIAARFNRYFFDSW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	11						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPRGTSTIAARFNRYFFDSW	21	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	9						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CAKAPRATSTIAARFNRYFFDAW	22	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPGGTSTIAARFNRYFFDSW	22	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	7						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CARAPPGTSTIAARFNRYFFDSW	24	IGKV3-20*01	IGKJ2*01	CHQYGSSDNTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVTAPDDTETIPARHNRYYFDSW	16	IGKV3-20*01	IGKJ2*01	CHQYGSSPNTF	8						
V3	IGHV1-69*06	IGHD6-6*01	IGHJ4*02	CVTAPDDTETIPARHNRYYFDSW	16	IGKV3-20*01	IGKJ2*01	CHQYGSSPNTF	8						

Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V1	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTMGVVLPFDYW	9	IGKV1-5*03	IGKJ1*01	CQQFNSFSPWTF	6						
V1	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTMGVVLPFDYW	9	IGKV1-5*03	IGKJ1*01	CQQFNSFSPWTF	6						
V1	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTMGVVLPFDYW	9	IGKV1-5*03	IGKJ1*01	CQQFNSFSPWTF	6						
V1	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTMGVVLPFDYW	8	IGKV1-5*03	IGKJ1*01	CQQFNSFSPWTF	7						
V1	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTMGVVLPFDYW	8	IGKV1-5*03	IGKJ1*01	CQQFNSFSPWTF	6	2,05E+03	6,01E-04	2,94E-07			
V3	IGHV2-5*10	IGHD3-3*02	IGHJ4*02	CARTVGVVLPFDYW	9	IGKV1-5*03	IGKJ1*01	CQQFNRNSPWTF	7	6,66E+05	5,36E-05	8,05E-11			
V2	IGHV2-5*10	IGHD3-10*01	IGHJ4*02	CARTVRGVVPFDYW	12	IGKV1-5*03	IGKJ1*01	CQEYNSDSRTF	8	1,46E+04	1,37E-05	9,34E-10			
V3	IGHV2-5*10	IGHD3-10*01	IGHJ4*02	CARTVRGVVPFDYW	11	IGKV1-5*03	IGKJ1*01	CQQYNSDSRTF	8						
V3	IGHV2-5*10	IGHD3-10*01	IGHJ4*02	CARTVGAFGRFPFDFW	7	IGKV1-5*03	IGKJ4*01	CQQYNSDSLTF	10						
V3	IGHV2-5*01	IGHD3-10*02	IGHJ4*02	CARHIRGVTPFDYR	8	IGKV1-5*03	IGKJ1*01	CQQYDSYFWTF	6						
V1	IGHV2-5*01	IGHD6-13*01	IGHJ4*02	CARTVASLGTAFDYW	13	IGKV1-9*01	IGKJ1*01	CQQLNSYPTF	7	2,77E+05	1,09E-04	3,94E-10			
V1	IGHV2-5*04	IGHD2-2*02	IGHJ4*02	CARSVVPATRAFDFW	20	IGKV1-5*03	IGKJ1*01	CQHYDGNSLTF	12	1,70E+05	1,60E-04	9,39E-10			
V2	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARSVVPATRAFDFW	22	IGKV1-5*03	IGKJ1*01	CQQYDGNSLTF	10	4,19E+05	5,19E-06	1,24E-11			
V3	IGHV2-5*10	IGHD2-2*02	IGHJ4*02	CARSVVPATRAFDFW	17	IGKV1-5*03	IGKJ1*01	CQHYDGNSLTF	10	5,79E+04	9,21E-05	1,59E-09			
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARSVVPATRAFDFW	16	IGKV1-5*03	IGKJ1*01	CQHYDGNSLTF	10						
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARSVVPATRAFDFW	20	IGKV1-5*03	IGKJ1*01	CQQYDGNSLTF	10						
V3	IGHV2-5*04	IGHD2-15*01	IGHJ4*02	CARAGVPATRSFDFW	16	IGKV1-5*03	IGKJ1*01	CQQYDGHSLTF	12						
V3	IGHV2-5*10	IGHD2-2*02	IGHJ4*02	CARSVVPATRSFDYW	12	IGKV1-5*03	IGKJ1*01	CQQYDGNSLTF	9						
V2	IGHV1-69*01	IGHD2-21*02	IGHJ6*02	CARDSRTRARGSGGFYRGNVYYYAMDVW	20	IGKV3-20*01	IGKJ4*01	CQQNGRSPLSF	13						
V3	IGHV1-69*01	IGHD6-19*01	IGHJ6*02	CARDSRTRARGSGGWYRGNVYYYAMDVW	21	IGKV3-20*01	IGKJ4*01	CQQNGRSPLTF	7						
V2	IGHV2-26*01	ND	IGHJ6*03	CARMRRIFINHYSSYMDVW	13	IGKV3-20*01	IGKJ4*01	CQQHGRPPLTF	7						
V3	IGHV2-26*01	ND	IGHJ6*03	CARMRRIFINHYSSYMDVW	15	IGKV3-20*01	IGKJ4*01	CQQHGRPPLTF	10	1,66E+04	4,68E-04	2,82E-08	4,18E+04	2,95E-04	7,06E-09
V3	IGHV2-26*01	ND	IGHJ6*03	CARMRRIFINHYSSYMDVW	15	IGKV3-20*01	IGKJ4*01	CQQHGRPPLTF	8						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	18	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	7						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	17	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	7						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	16	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	7						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	16	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	7						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	15	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	7	4,08E+04	2,09E-04	5,14E-09			
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	15	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	8						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYW	15	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	8						
V1	IGHV2-5*10	IGHD1-26*01	IGHJ4*02	CARIVGTHGFDYC	17	IGKV1-9*01	IGKJ2*01	CQQLNRYPPYTF	8	1,83E+05	1,39E-03	7,61E-09			
V1	IGHV4-61*08	IGHD5-12*01	IGHJ4*02	CARARRTYSGYDSAFDYW	13	IGKV1-39*01	IGKJ2*01	CQQSFGTPYTF	9	1,08E+06	1,63E-04	1,51E-10			
V1	IGHV4-61*08	IGHD5-12*01	IGHJ4*02	CARARRTYSGYDSAFDYW	13	IGKV1-39*01	IGKJ2*01	CQQSFGTPYTF	10						
V1	IGHV4-61*08	IGHD5-12*01	IGHJ4*02	CARARRTYSGYDSAFDYW	14	IGKV1-39*01	IGKJ2*01	CQQSFGTPYTF	9						
V3	IGHV4-61*01	IGHD5-12*01	IGHJ4*02	CARARRTFSGYDSAFDYW	14	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	7	5,09E+04	2,11E-04	4,14E-09			
V3	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	CARARRTFSGYYSAFDYW	13	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	7						
V1	IGHV4-61*01	IGHD2-2*02	IGHJ6*02	CARIKDVGHCSGGSCYSGAGWFDPW	3	IGKV3-20*01	IGKJ1*01	CQQSGSSPLTF	1						
V3	IGHV4-30-4*01	IGHD1-7*01	IGHJ4*02	CARGDKWAFWFGEIDYW	4	IGKV4-1*01	IGKJ4*01	CQQYYSTPLTF	6	6,14E+04	1,47E-05	2,39E-10	3,67E+04	3,16E-05	8,61E-10
V3	IGHV4-39*01	ND	IGHJ5*02	CARQTDNWFDPW	16	IGKV3-15*01	IGKJ2*01	CQQYKNWPPYTF	3						
V3	IGHV4-39*01	ND	IGHJ5*02	CARQTDNWFDPW	17	IGKV3-15*01	IGKJ2*01	CQQYKNWPPYTF	3	1,63E+03	1,18E-04	7,24E-08			
V1	IGHV4-61*01	IGHD3-10*01	IGHJ3*02	CARDVRRRFGEFLRPFDLW	16	IGKV1-16*01	IGKJ4*01	CHQYNTYPPTF	6	7,32E+04	3,77E-04	5,15E-09			
V1	IGHV4-61*08	IGHD3-10*01	IGHJ3*02	CARDVRRRFGEFFRPFDLW	17	IGKV1-16*01	IGKJ4*01	CHQYNTYPPTF	7						
V1	IGHV4-61*08	IGHD3-10*01	IGHJ3*02	CARDVRRRFGEFFRPFDLW	17	IGKV1-16*01	IGKJ4*01	CHQYNTYPPTF	6						
V1	IGHV4-61*08	IGHD3-10*01	IGHJ3*02	CARDVRRRFGEFFRPFDLW	18	IGKV1-16*01	IGKJ4*01	CHQYNTYPPTF	6						
V2	IGHV4-61*08	IGHD3-10*01	IGHJ3*02	CARDIRRRFGEFFRPFDFW	20	IGKV1-16*01	IGKJ4*01	CHQYNTYPRTF	10	4,60E+05	4,01E-05	8,71E-11			
V3	IGHV4-61*08	IGHD3-10*01	IGHJ3*02	CARDIRRRFGEFFRPFDFW	20	IGKV1-16*01	IGKJ4*01	CHQYNTYPRTF	9	7,16E+04	1,14E-04	1,59E-09			
V2	IGHV4-61*01	IGHD5-5*01	IGHJ4*02	CARSGPEYSYGYTFDYW	0	IGKV1-5*03	IGKJ1*01	CQQYNSYSGTF	0						
V2	IGHV4-34*01	IGHD2-2*03	IGHJ4*02	CARGHFKEEPTLPTMSRPTTPPYFDYW	0	IGKV3-11*01	IGKJ4*01	CQQRSNWPLTF	7						
V2	IGHV3-48*01	IGHD1-26*01	IGHJ3*02	CTRDLYTGSPVEAFDIW	19	IGKV1-6*01	IGKJ1*01	CLHDNGYPQTF	7						
V2	IGHV3-49*03	IGHD3-10*01	IGHJ4*02	CTRDREGSGSSKPPGPPHFDYW	0	IGKV1D-33*01	IGKJ3*01	CQQYDNLPPVTF	0						
V2	IGHV3-30*04	IGHD2-15*01	IGHJ3*02	CARDEDGVAGAFDIW	0	IGKV1-5*03	IGKJ2*01	CQQYNSYPYTF	0	1,77E+02	3,03E-05	1,71E-07	6,53E+04	8,77E-04	1,34E-08
V2	IGHV1-69*06	IGHD3-22*01	IGHJ6*02	CARADHYDSSGSNFFFYGMDVW	8	IGKV3-11*01	IGKJ3*01	CQQRSNWPPLFSF	4						
V2	IGHV4-34*01	IGHD2-2*02	IGHJ6*02	CARGPCRSTSCPSYYYGMDVW	0	IGKV1-39*01	IGKJ1*01	CQQSYSTLRAF	0						
V3	IGHV7-4-1*02	IGHD3-22*01	IGHJ4*02	CARASYHYDSSGSSPQYYFDSW	13	IGKV1-39*01	IGKJ4*01	CQQSYGTLLTF	4	1,48E+04	1,93E-04	1,30E-08			
V3	IGHV1-46*01	IGHD5-5*01	IGHJ6*02	CARDVDLWLTADKGDYYGMDFW	10	IGKV1-16*01	IGKJ5*01	CQRSKTYPPVWRLTN	11						
V3	IGHV5-51*01	IGHD3-9*01	IGHJ4*02	CARVYYDW	16	IGKV3-15*01	IGKJ1*01	CQQYNNWPPATF	5	3,31E+03	5,38E-04	1,63E-07	7,55E+03	6,08E-04	8,05E-08
V3	IGHV3-49*03	ND	IGHJ5*02	CTRLQYCSYVS	5	IGKV2-28*01	IGKJ3*01	CMQALQSPPFTF	3	1,93E+05	1,12E-05	5,79E-11	1,77E+05	5,70E-05	3,23E-10
V3	IGHV4-59*01	ND	IGHJ6*03	CARVMKVGDYYFYYMDVW	13	IGKV3-11*01	IGKJ1*01	CQQRRFWPWTF	5						
V3	IGHV3-21*01	IGHD2-21*02	IGHJ4*03	CARDPRARGLLFGNFDYW	12	IGKV3-15*01	IGKJ2*01	CQQYNAWPPYTF	8						
V3	IGHV3-15*02	IGHD7-27*01	IGHJ5*02	CTTSAGALAGPGILW	13	IGKV3-20*01	IGKJ4*02	CQQYSISHPLTF	11						
V3	IGHV4-59*01	IGHD2-2*02	IGHJ4*02	CARGGPSWTVIDHW	12	IGKV1-39*01	IGKJ3*01	CQQSYSAPLF	7						
V3	IGHV4-30-2*01	IGHD3-16*01	IGHJ3*02	CARSSAYVYAFDIW	12	IGKV1-39*01	IGKJ2*01	CQHSSSTPYTF	4						
V3	IGHV3-74*01	IGHD2-15*01	IGHJ3*02	CASGDCNSGSCYFRDAFDIW	5	IGKV4-1*01	IGKJ2*01	CQQYKSIPYTF	2	2,16E+03	4,11E-05	1,91E-08	7,10E+03	2,11E-04	2,97E-08
V3	IGHV1-69*01	ND	IGHJ6*03	CARGVGTAGTGMDVW	10	IGKV3-15*01	IGKJ3*01	CQNYNDWPRIFTF	5						
V3	IGHV4-34*12	IGHD4-17*01	IGHJ5*02	CAQGVVGSW	18	IGKV4-1*01	IGKJ2*01	CQQYYTTPYTF	8	2,36E+05	4,58E-05	1,94E-10			
V3	IGHV2-5*04	IGHD2-2*02	IGHJ4*02	CARAVVPARVPFDFW	13	IGKV1-5*03	IGKJ5*01	CQHSNTYVF	11						
V3	IGHV4-39*01	IGHD5-12*01	IGHJ5*02	CGRQDATIRLNGPIDLW	12	IGKV1-5*03	IGKJ2*02	CQQYNTYYSTF	9						
V3	IGHV3-23*01	IGHD6-19*01	IGHJ3*02	CAKYRRQWLSNECFDIW	12	IGKV3-20*01	IGKJ3*01	CQQYVSSPLTF	7						
V3	IGHV2-5*10	IGHD3-10*01	IGHJ4*02	CARSMRMVRGRIEQFDYW	10	IGKV1-9*01	IGKJ5*01	CQHLNNNLITF	4						
V3	IGHV1-2*02	IGHD1-26*01	IGHJ4*02	CARGVFSRANYGFLYSFDSW	24	IGKV1-39*01	IGKJ4*01	CQQTFPTSVSF	6						
V3	IGHV1-46*01	IGHD2-2*03	IGHJ4*02	CARDPKVWSASSGFDYW	17	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF	5						
V3	IGHV3-15*01	IGHD2-2*02	IGHJ4*02	CTARYCTRTSCYGTPDYW	9	IGKV1-5*03	IGKJ1*01	CQQYNSYSLWTF	7						
V3	IGHV3-30*18	IGHD6-19*01	IGHJ4*02	CAKGITRPGVAVRERFDHW	14	IGKV1-39*01	IGKJ4*01	CQQSYNTPFTF	14						
V3	IGHV2-5*01	IGHD7-27*01	IGHJ5*02	CARKAPGWGPAFDWFGPW	13	IGKV1-39*01	IGKJ1*01	CQQSHTFPWTF	18						
V3	IGHV3-74*01	IGHD7-27*01	IGHJ3*02	CARLGLIW	11	IGKV1-39*01	IGKJ2*01	CQQNYNTPYTF	13						
V3	IGHV3-23*01	IGHD6-19*01	IGHJ3*02	CAKHAMGGWYGFGAFDIW	14	IGKV1-5*03	IGKJ1*01	CQHYDTYSPTF	8						
V3	IGHV3-21*01	ND	IGHJ4*02	CVSGGSLDYW	7	IGKV1-39*01	IGKJ1*01	CLQTFTQSRTF	13						
V3	IGHV2-26*01	IGHD3-3*01	IGHJ4*02	CARVRGRRIPLFGGTIIRGARFDYW	16	IGKV1D-33*01	IGKJ3*01	CQQYDYLPDSAF	5	2,08E+05	2,38E-05	1,14E-10			
V3	IGHV3-74*01	IGHD3-16*02	IGHJ3*01	CARKLRVWGPFRLDAFDVW	10	IGKV1-39*01	IGKJ4*02	CHQSYITPLTF	10						
V3	IGHV3-48*01	IGHD2-21*02	IGHJ3*02	CVRVLRWNNDAFHIW	16	IGKV3-20*01	IGKJ2*01	CQQYGSSPYTF	11						
V3	IGHV4-59*01	IGHD5-24*01	IGHJ3*01	CARSREPYDMKAFDVW	14	IGKV4-1*01	IGKJ2*03	CQQYLNTPYIF	10						
V3	IGHV3-23*01	IGHD5-12*01	IGHJ4*02	CAKAGIQWLRDYFYDSW	12	IGKV1-5*03	IGKJ1*01	CQPYNGDSPTF	6						
V3	IGHV3-64*05		IGHJ5*02	CVKDSGVTPSVW	5	IGKV4-1*01	IGKJ1*01	CQQYYSSLPWTF	1	1,89E+04	3,07E-05	1,63E-09			
V3	IGHV4-59*02	IGHD6-19*01	IGHJ4*02	CAREKESAGWNAHYFDYW	30	IGKV1-5*03	IGKJ1*01	CQQYYSYPWA	5	3,84E+05	1,74E-05	4,53E-11	5,76E+04	1,97E-04	3,43E-09

Supplementary table 1b. Repertoire and affinities of antibody clonotypes from donor TT2

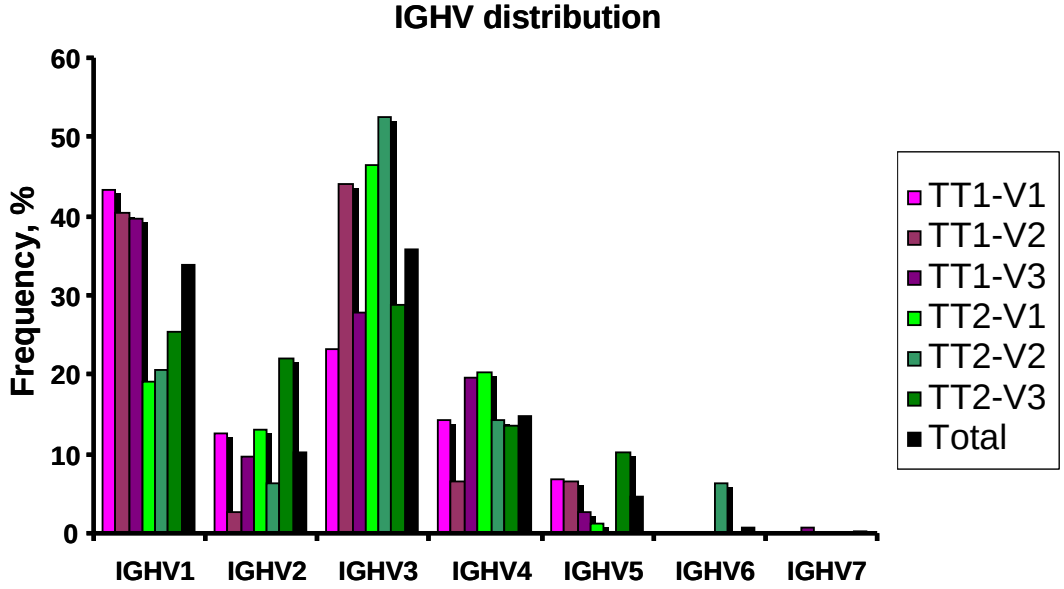
Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYYFDYW	10	IGKV1-39*01	IGKJ2*01	CQESYKIPYTF	6						
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYYFDYW	10	IGKV1-39*01	IGKJ2*01	CQESYKIPYTF	6	1,31E+06	1,21E-04	9,25E-11			
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYYFDYW	9	IGKV1-39*01	IGKJ2*01	CQESYKIPYTF	6	2,72E+05	1,44E-04	5,27E-10	9,45E+04	7,00E-04	7,41E-09
V2	IGHV1-18*01	IGHD3-16*01	IGHJ4*02	CARDHSSPYYFDYW	9	IGKV1-39*01	IGKJ2*01	CQESYKIPYTF	6						
V2	IGHV1-18*01	IGHD4-4*01	IGHJ4*02	CARDYSSPYYFDYW	9	IGKV1-39*01	IGKJ2*01	CQESYKIPYTF	7						
V1	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGPIYYFDHW	17	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	7						
V1	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGPIYYFDHW	17	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	7						
V1	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYFGSGPIYYFDHW	16	IGKV1-39*01	IGKJ2*01	CQQSYKTPYTF	7						
V1	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDYYGSGSHYYFDYW	9	IGKV1-39*01	IGKJ2*02	CQQIYSTPRTF	10	3,31E+07	8,94E-05	2,70E-12			
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDWNQGSGIYYISDW	8	IGKV1-39*01	IGKJ2*01	CQQTYRTPYTF	7	1,82E+05	2,54E-05	1,40E-10	2,03E+06	1,95E-04	9,61E-11
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDWNQGSGIYYISDW	8	IGKV1-39*01	IGKJ2*01	CQQTYRTPYTF	7						
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDWNQGSGIYYISDW	8	IGKV1-39*01	IGKJ2*01	CQQTYRTPYTF	8						
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDWNQGSGIYYISDW	8	IGKV1-39*01	IGKJ2*01	CQQTYRTPYTF	8	1,21E+05	4,26E-05	3,53E-10	1,35E+05	2,22E-04	1,64E-09
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARDWNQGSGIYYISDW	8	IGKV1-39*01	IGKJ2*01	CQQTYRTPYTF	8						
V2	IGHV1-8*01	ND	IGHJ5*02	CARGAGAQGYDWFDPW	6	IGKV1-17*01	IGKJ1*01	CLQCDNTGRSAKGPRW	5						
V1	IGHV1-2*02	IGHD2-2*02	IGHJ4*02	CATWVSRESSFDYW	12	IGKV3-11*01	IGKJ2*01	CQQRSYWYTF	4						
V2	IGHV1-3*01	IGHD4-17*01	IGHJ1*01	CARVSRYGDYGPEYFQHW	10	IGKV2-28*01	IGKJ1*01	CVQGLQSRTF	1						
V1	IGHV1-46*01	IGHD2-2*03	IGHJ6*03	CARQYCSSASCYASLRHSGQFYYFMDVW	18	IGKV3-20*01	IGKJ5*01	CQERITF	6						
V1	IGHV1-46*01	IGHD2-2*03	IGHJ6*03	CARQYCSSASCYVSLRHSGQFYYFMDVW	18	IGKV3-20*01	IGKJ5*01	CQERITF	6						
V1	IGHV1-46*01	IGHD2-2*03	IGHJ6*03	CARQYCSSASCYVSLRHSGQFYYFMDVW	18	IGKV3-20*01	IGKJ5*01	CQERITF	7						
V3	IGHV1-46*01	IGHD3-10*01	IGHJ5*02	CARATLTDWSGRFWFDPW	7	IGKV3-20*01	IGKJ3*01	CQQYDDSGHFTF	8						
V1	IGHV3-21*01	ND	IGHJ6*03	CARYLGSTRGYYYMDVW	18	IGKV3-20*01	IGKJ3*01	CQQYGSSPPITF	6	7,49E+04	2,09E-04	2,79E-09			
V1	IGHV3-21*01	ND	IGHJ6*03	CARYLGSTRGYYYMDVW	19	IGKV3-20*01	IGKJ3*01	CQQYGSSPPITF	6						
V2	IGHV3-21*01	ND	IGHJ6*03	CARYRGGTRGYYYMDVW	13	IGKV3-20*01	IGKJ3*01	CQQYASSPPITF	8	3,63E+06	8,72E-06	2,40E-12			
V2	IGHV3-21*01	ND	IGHJ6*03	CARYRGGTRGYYYMDVW	14	IGKV3-20*01	IGKJ3*01	CQQYASSPPITF	8						
V2	IGHV1-69*01	IGHD1-14*01	IGHJ6*02	CARARYSYYDSSGYYRLDFW	6	IGKV1-9*01	IGKJ3*01	CQQLNSPPLTF	4						
V1	IGHV1-69*08	IGHD2-2*02	IGHJ5*02	CARGKDCRANNCYLSERNNWFDPW	18	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	1	3,84E+05	3,97E-05	1,03E-10			
V2	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNNCYLSERNNWFDPW	17	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	2	3,49E+05	4,53E-05	1,30E-10	7,46E+05	1,50E-04	2,01E-10
V2	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNNCYLSERSNWFDPW	17	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	2						
V2	IGHV1-69*11	IGHD2-2*02	IGHJ5*02	CARGKDCRSNNCYLSERNNWFDPW	17	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	3						
V3	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNGCFLSERNNWFDPW	18	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	2	5,60E+05	2,48E-05	3,91E-11	1,13E+05	7,27E-05	6,45E-10
V3	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNGCFLSERNNWFDPW	18	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	8						
V3	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNGCFLSERNNWFDPW	18	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	3	2,76E+04	6,67E-05	2,42E-09	5,70E+04	5,18E-05	9,08E-10
V3	IGHV1-69*01	IGHD2-2*02	IGHJ5*02	CARGKDCRSNGCFLSERNNWFDPW	18	IGKV1-5*03	IGKJ4*01	CQQYNSYFLTF	3						
V1	IGHV1-69*01	IGHD6-6*01	IGHJ3*02	CARGVAGIASRRHAYDIW	12	IGKV1-39*01	IGKJ2*02	CQQSYSIPSTF	4						
V1	IGHV1-69*01	IGHD1-26*01	IGHJ4*02	CAKLPSGSYYDYFDSW	16	IGKV3-20*01	IGKJ4*01	CQHYGNSLAPPF	6						
V1	IGHV1-46*01	IGHD2-21*02	IGHJ6*02	CARLGIAARHYFYGVDVW	17	IGKV1-27*01	IGKJ1*01	CQKYNSAPLTF	4	8,01E+04	3,18E-03	3,97E-08			
V1	IGHV1-46*01	IGHD2-21*02	IGHJ6*02	CARLGIAARHYFYGVDVW	18	IGKV1-27*01	IGKJ1*01	CQKYNSAPLTF	4						
V1	IGHV1-24*01	IGHD3-3*01	IGHJ4*02	CTTVGEALITLFGTVIRRARDFDSW	6	IGKV1-16*01	IGKJ4*01	CQQYDGYPLTF	6						
V2	IGHV1-24*01	IGHD3-3*01	IGHJ4*02	CATVGETRFTLFGTLMRRPRELRDW	18	IGKV1-16*01	IGKJ4*01	CQQYDSYPLTF	8	4,53E+05	8,68E-05	1,92E-10			
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	6	IGKV3-20*01	IGKJ1*01	CQYQGPF	5						
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	6	IGKV3-20*01	IGKJ1*01	CQYQGPF	6						
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	7	IGKV3-20*01	IGKJ1*01	CQYQGPF	6						
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	7	IGKV3-20*01	IGKJ1*01	CQYQGPF	5						
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	7	IGKV3-20*01	IGKJ1*01	CQYQGPF	5	1,93E+05	3,17E-05	1,64E-10			
V3	IGHV5-51*01	IGHD3-3*01	IGHJ6*03	CARTQYDAWSGSVLEDIYYYYYMDVW	7	IGKV3-20*01	IGKJ1*01	CQYQGPF	5						
V1	IGHV5-51*01	IGHD6-6*01	IGHJ5*02	CARLMRYSSPSGRLEGLVGRFDPW	14	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	11						
V1	IGHV3-21*01	ND	IGHJ4*02	CASGSTLDYW	8	IGKV1-39*01	IGKJ1*01	CLQNYIPRRTF	9						
V1	IGHV3-21*01	ND	IGHJ4*02	CASGSTLDYW	7	IGKV1-39*01	IGKJ1*01	CLQNYIPRRTF	9						
V1	IGHV3-21*01	ND	IGHJ4*02	CVSGSSLDYW	11	IGKV1-39*01	IGKJ1*01	CLQTYSPRRTF	8						
V1	IGHV3-21*01	ND	IGHJ4*02	CVSGSSLDYW	11	IGKV1-39*01	IGKJ1*01	CLQTYSPRRTF	8						
V1	IGHV3-21*02	ND	IGHJ4*02	CVSGSSLDYW	12	IGKV1-39*01	IGKJ1*01	CLQTYSPRRTF	8						
V1	IGHV3-21*01	ND	IGHJ4*02	CVSGSSLDYW	12	IGKV1-39*01	IGKJ1*01	CLQTYSPRRTF	8						
V1	IGHV3-21*01	ND	IGHJ4*02	CATGVTMDYW	13	IGKV3-20*01	IGKJ1*01	CQQYGSLPRTF	4	1,41E+03	6,72E-04	4,78E-07			
V2	IGHV3-21*01	IGHD4-23*01	IGHJ4*02	CATGNTLDYW	10	IGKV3-20*01	IGKJ1*01	CHQYFSSPRTF	8						
V2	IGHV3-21*01	ND	IGHJ4*02	CATGNTLDYW	10	IGKV3-20*01	IGKJ1*01	CHQYFSSPRTF	8						
V2	IGHV3-21*01	ND	IGHJ4*02	CATGNTLDYW	11	IGKV3-20*01	IGKJ1*01	CHQYFSSPRTF	8						
V2	IGHV3-21*01	ND	IGHJ4*02	CTSGVTFDYW	16	IGKV3-20*01	IGKJ2*02	CRQYGSSPTTF	10	3,75E+05	1,01E-04	2,68E-10			
V2	IGHV3-21*01	ND	IGHJ4*02	CTSGVTFDYW	17	IGKV3-20*01	IGKJ2*02	CRQYGSSPTTF	10						
V2	IGHV3-21*01	ND	IGHJ4*02	CTSGVTFDYW	17	IGKV3-20*01	IGKJ2*02	CRQYGSSPTTF	11						
V2	IGHV3-21*01	IGHD1-26*01	IGHJ1*01	CVNGDYVV	10	IGKV2-30*01	IGKJ1*01	CMQGTHWPRTF	2						
V3	IGHV3-21*01	IGHD4-17*01	IGHJ4*02	CVNGDYVV	7	IGKV2-30*01	IGKJ1*01	CMQGTHWPRTF	5	3,68E+05	9,29E-06	2,52E-11	7,02E+05	1,48E-05	2,11E-11
V3	IGHV3-21*01	IGHD2-21*02	IGHJ4*02	CVNGDYVV	14	IGKV2-30*01	IGKJ1*01	CMQGTHWPRTF	3						
V1	IGHV3-21*01	ND	IGHJ4*02	CVTGSSHDFW	15	IGKV1-39*01	IGKJ1*01	CQQTYSDIRTF	7						
V1	IGHV3-21*01	IGHD6-25*01	IGHJ3*01	CARDRAQIWGYRRGGDALDVW	13	IGKV3-20*01	IGKJ1*01	CHQYGSSPPWTF	3						
V2	IGHV3-11*01	IGHD7-27*01	IGHJ2*01	CARRPNLWGSALWYFDLW	12	IGKV1-9*01	IGKJ5*01	CQQLNSFPTF	4						
V3	IGHV3-11*01	IGHD7-27*01	IGHJ2*01	CARRPNLWGSALWYFDLW	16	IGKV1-9*01	IGKJ5*01	CQQLNSFPTF	7	1,08E+05	4,75E-04	4,39E-09	5,90E+04	7,79E-04	1,32E-08
V1	IGHV3-23*01	IGHD3-3*02	IGHJ3*01	CVKYLWGGYAIDVW	10	IGKV1-39*01	IGKJ1*01	CQQSYITPWTF	14						
V1	IGHV3-23*01	IGHD3-3*02	IGHJ6*02	CVKYLWGGYAIDVW	10	IGKV1-39*01	IGKJ1*01	CQQSYITPWTF	13	8,14E+05	1,57E-04	1,93E-10			
V1	IGHV3-23*01	IGHD4-17*01	IGHJ6*02	CAKYLSGGYAIDVW	13	IGKV1-39*01	IGKJ1*01	CQQSYITPWTF	11	4,29E+06	2,51E-04	5,84E-11			
V2	IGHV3-23*01	IGHD2-8*02	IGHJ6*02	CAKYLSGGYAIDVW	20	IGKV1-39*01	IGKJ1*01	CQQSYITPWTF	11						

Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAEGLIFGVAAYYFDHW	5	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	4						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVAAYYFDHW	5	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	4						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVAAYYFDHW	5	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	4						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVAAYYFDYW	6	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	6						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVAAYYFDYW	7	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	4	**5,19E+05**	**1,38E-04**	**2,66E-10**			
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVPAYYFDSW	9	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	2						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKDLILGVPHYYFDSW	9	IGKV3-11*01	IGKJ1*01	CQHRANWPRTF	7						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKDLILGVPHYYFDSW	10	IGKV3-11*01	IGKJ1*01	CQHRANWPRTF	7						
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKDLILGVPHYYFDSW	10	IGKV3-11*01	IGKJ1*01	CQHRANWPRTF	6	**4,45E+05**	**2,65E-04**	**5,95E-10**			
V1	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKGLIFGVPAYYFDSW	11	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	3	**2,79E+05**	**1,01E-03**	**3,62E-09**			
V2	IGHV3-23*01	IGHD3-3*02	IGHJ4*02	CAKGLIFGVAAYYFDSW	16	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	6			**3,53E-10**			
V3	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKSLIFGVAAYYFDSW	15	IGKV3-11*01	IGKJ4*01	CQHRGNWPRTF	4						
V3	IGHV3-23*01	IGHD3-3*01	IGHJ4*02	CAKSLIFGVAAYYFDSW	15	IGKV3-11*01	IGKJ4*01	CQHRGNWLLTF	9						
V3	IGHV3-23*01	IGHD2-2*02	IGHJ4*02	CAKADNIVLVPAALTRPVDYW	13	IGKV4-1*01	IGKJ1*01	CQQYYTTPWTF	4	**1,17E+05**	**3,13E-05**	**2,68E-10**			
V3	IGHV3-23*01	IGHD2-2*02	IGHJ4*02	CAKADNIVLVPAALTRPVDYW	13	IGKV4-1*01	IGKJ1*01	CQQYYTTPWTF	5						
V1	IGHV3-48*02	IGHD5-24*01	IGHJ6*03	CARGVLGGYNNGLYYYYYLDVW	13	IGKV3-20*01	IGKJ5*01	CQQCGSSPYAF	3						
V1	IGHV3-48*02	IGHD3-3*02	IGHJ4*02	CARDLFFGPLKGLFDSW	6	IGKV3-20*01	IGKJ1*01	CQQYHNSPWTF	7						
V1	IGHV3-48*02	IGHD3-3*02	IGHJ4*02	CARDLFFGPLKGLFDSW	5	IGKV3-20*01	IGKJ1*01	CQQYHNSPWTF	6						
V1	IGHV3-23*01	IGHD6-19*01	IGHJ4*02	CAKGRKQWLVPDFDSW	13	IGKV1-12*01	IGKJ5*01	CQQTKTFPLTF	10	**1,10E+05**	**4,65E-04**	**4,22E-09**			
V1	IGHV3-74*01	IGHD2-8*02	IGHJ6*03	CVRPRGYCADGLCYPALYFYYMDVW	4	IGKV1-27*01	IGKJ3*01	CQKYNSVPLTF	4						
V2	IGHV3-30*18	IGHD2-2*03	IGHJ6*01	CAKGLSQALNYYGSGSPFL	11	IGKV1-9*01	IGKJ2*01	CQQLNSYPYTF	10						
V2	IGHV3-30*18	ND	IGHJ4*02	CAKDRITAADYW	13	IGKV1-39*01	IGKJ1*01	CQQSYTIWWTF	13						
V2	IGHV3-33*01	IGHD2-8*01	IGHJ4*02	CARERGHDTNGQPDNW	8	IGKV1-39*01	IGKJ2*02	CQQSYSPPFTF	10	**3,23E+05**	**9,40E-05**	**2,90E-10**			
V2	IGHV3-33*01	IGHD2-8*01	IGHJ4*02	CARERGHDTNGQPDNW	8	IGKV1-39*01	IGKJ2*02	CQQSYSPPFTF	10						
V2	IGHV3-33*01	IGHD3-10*02	IGHJ5*02	CARARGPYSGTYYATGWW	11	IGKV1D-33*01	IGKJ3*01	CQQYHNLPLTF	10						
V2	IGHV3-33*01	IGHD3-10*02	IGHJ5*02	CARARGPYSGTYYATGWW	11	IGKV1-39*01	IGKJ2*01	CQQSFSTLYTF	6						
V1	IGHV3-33*01	IGHD3-10*02	IGHJ4*02	CARGSGTYSFLDNW	7	IGKV1-17*01	IGKJ1*01	CLQHNSYPPTF	7	**3,96E+04**	**3,58E-04**	**9,03E-09**			
V1	IGHV3-33*01	IGHD6-25*01	IGHJ3*02	CARDSAPLSRRGALGIW	15	IGKV1-6*01	IGKJ2*01	CLQDYNYPYSF	3	**3,20E+03**	**7,96E-04**	**2,49E-07**			
V1	IGHV3-33*01	IGHD6-25*01	IGHJ3*02	CARDSAPLSRRGALGIW	16	IGKV1-6*01	IGKJ2*01	CLQDYNYPYSF	4						
V1	IGHV3-30*18	IGHD3-3*01	IGHJ6*03	CGKDHIGYNSQSGYRPESYYYMDLW	11	IGKV3-11*01	IGKJ4*01	CQQRNNWPPLTF	6						
V1	IGHV3-30*18	IGHD3-3*01	IGHJ6*03	CGKDHIGYNSRSGYRPESYYYMDLW	11	IGKV3-11*01	IGKJ4*01	CQQRNNWPPLTF	5						
V1	IGHV3-30*18	IGHD3-3*01	IGHJ6*03	CGKDHVGYNSRSGYRPESYYYMDLW	11	IGKV3-11*01	IGKJ4*01	CQQRNNWPPLTF	5						
V1	IGHV3-7*01	IGHD6-13*01	IGHJ4*03	CARAGSSWSLRPTTFDYW	14	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7						
V1	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARASSSWSLRPTTFDYW	14	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7						
V2	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	14	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7						
V2	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	14	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7	**1,21E+06**	**1,47E-04**	**1,22E-10**	**6,57E+05**	**7,35E-04**	**1,12E-09**
V1	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7	**9,07E+05**	**1,45E-04**	**1,60E-10**			
V2	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7	**2,89E+05**	**1,27E-04**	**4,38E-10**	**1,30E+06**	**7,01E-04**	**5,40E-10**
V2	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGTSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7						
V3	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	FQQFYHYPRTF	8	**8,93E+05**	**2,29E-04**	**2,56E-10**			
V3	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	8						
V3	IGHV3-7*01	IGHD6-13*01	IGHJ4*02	CARAGSSWSLRPTTFDYW	13	IGKV1-6*01	IGKJ1*01	CQQFYHYPRTF	7						
V1	IGHV3-7*01	IGHD3-16*02	IGHJ5*02	CARDGVITLGGVIELRWYDPW	16	IGKV1-9*01	IGKJ4*01	CQQLHSYPFTF	8	**1,70E+03**	**5,58E-04**	**3,28E-07**			
V2	IGHV3-7*01	IGHD5-24*01	IGHJ4*02	CARSTHSSADYW	9	IGKV3-11*01	IGKJ1*01	CQQRSHWPPTWTF	10						
V2	IGHV3-15*01	IGHD3-3*01	IGHJ4*02	CTATYYDFWSGLSRGGYW	7	IGKV1-5*01	IGKJ4*01	CQQYNAYSALTF	5						
V2	IGHV3-15*01	IGHD3-3*01	IGHJ4*02	CTATYYDFWSGLSRGGYW	7	IGKV1-5*03	IGKJ1*01	CQQYNSFPWTF	9						
V2	IGHV3-49*03	IGHD2-21*02	IGHJ6*02	CARGCPEIAVRAIYYYGMDVW	10	IGKV1-39*01	IGKJ1*01	CQHSYTTPWSF	5						
V1	IGHV4-59*01	IGHD2-2*02	IGHJ3*02	CARRHYCSSTSCYDAFDIW	10	IGKV1-39*01	IGKJ2*01	CQQTFSTLYTF	4	**2,66E+05**	**1,12E-04**	**4,21E-10**			
V2	IGHV4-59*01	IGHD2-2*02	IGHJ3*02	CARRHYCRSTSCYDAFDIW	13	IGKV1-39*01	IGKJ2*01	CQQSFSTLYTF	6						
V2	IGHV4-59*01	IGHD2-2*02	IGHJ3*02	CARRHYCRSTSCYDAFDIW	13	IGKV1-39*01	IGKJ2*01	CQQSFSTLYTF	7						
V2	IGHV4-59*01	IGHD2-2*02	IGHJ3*02	CARRHYCRSTSCYDAFDTW	13	IGKV1D-33*01	IGKJ3*01	CQQYHNLPLTF	10	**4,39E+04**	**1,00E-04**	**2,28E-09**			
V1	IGHV4-59*01	IGHD6-6*01	IGHJ5*02	CAREEFTSSSRWFDPW	14	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	6						
V1	IGHV4-59*01	IGHD6-6*01	IGHJ5*02	CAREEFTSSSRWFDPW	13	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	5						
V2	IGHV4-59*01	IGHD6-6*01	IGHJ5*02	CAREEFTSSSRWFDPW	13	IGKV1-39*01	IGKJ2*01	CQQSYSTPYTF	5	**5,83E+05**	**2,36E-05**	**4,04E-11**			
V1	IGHV4-59*01	IGHD3-3*02	IGHJ5*02	CARVRINLYGALLLSWFDSW	20	IGKV1-39*01	IGKJ1*01	CQQSDIIPWTF	6						
V1	IGHV4-59*01	IGHD3-3*01	IGHJ2*01	CARVRVSLIGVVVHRYFDLW	17	IGKV3-20*01	IGKJ4*01	CQQYYSSLTF	12						
V1	IGHV4-59*01	IGHD3-3*02	IGHJ2*01	CVRERVRVYGEIVHKYFDLW	14	IGKV1-39*01	IGKJ4*02	CQHRYDTATVTF	12						
V1	IGHV4-59*01	IGHD3-3*02	IGHJ2*01	CVRERVRVYGEIVHKYFDLW	13	IGKV3-15*01	IGKJ2*01	CQQYNNWPTLYTF	0						
V1	IGHV4-59*01	IGHD3-3*02	IGHJ2*01	CARERVRVYGEIIHKYFDLW	11	IGKV1-39*01	IGKJ4*01	XQQRYSTATVTF	9						

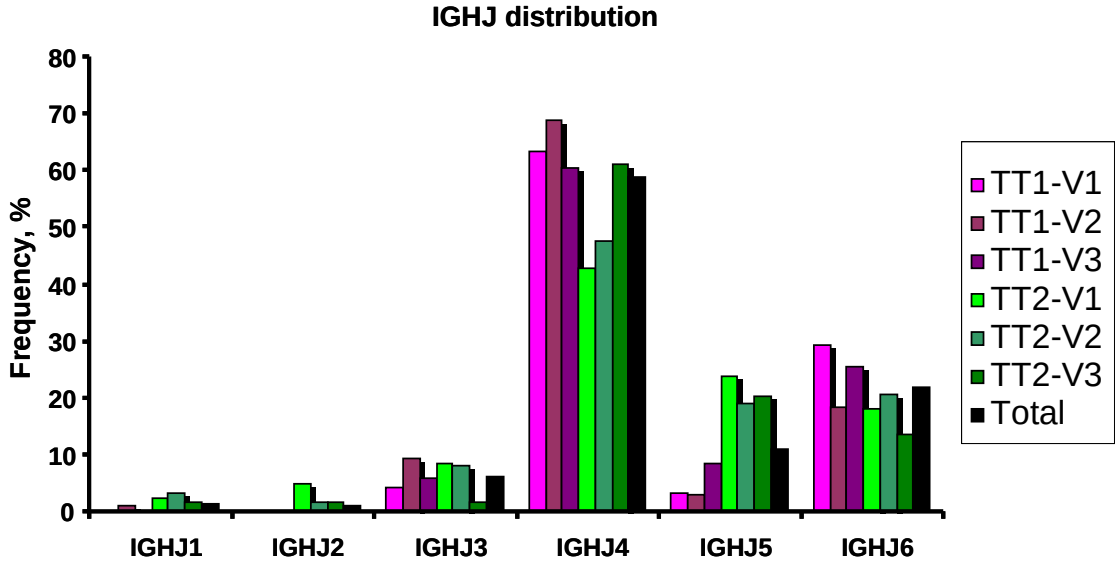
Repertoire	VH gene	D gene	JH gene	H-CDR3	# of SHM, H	VK gene	JK gene	K-CDR3	# of SHM, L	On-rate, 25C	Off-rate, 25C	Calc. KD, 25C, M	On-rate, 37C	Off-rate, 37C	Calc. KD, 37C, M
V2	IGHV3-13*01	IGHD4-17*01	IGHJ6*02	CARAKQGHTGLDVW	8	IGKV2-28*01	IGKJ2*02	CMQALQTPRTF	4						
V1	IGHV1-8*01	ND	IGHJ5*02	CAVVAENWFDPW	20	IGKV3-15*01	IGKJ2*01	CQQYRNWPPYTF	6						
V1	IGHV1-8*01	ND	IGHJ5*02	CAVVAENWFDPW	20	IGKV3-15*01	IGKJ2*01	CQQYRNWPPYTF	6						
V1	IGHV4-4*02	IGHD2-21*02	IGHJ5*02	CARLPKHYIAEAVTW	12	IGKV1-5*03	IGKJ5*01	CQQYNSYPITF	10						
V1	IGHV4-4*02	IGHD2-21*02	IGHJ1*01	CARLPKHYIAEAVTW	12	IGKV1-5*03	IGKJ5*01	CQQYNSYPITF	8						
V1	IGHV4-4*02	IGHD2-21*02	IGHJ1*01	CARLPKHYIAEAVTW	10	IGKV1-5*03	IGKJ5*01	CQQYNSYPTTF	10	5,10E+04	7,54E-04	1,48E-08			
V3	IGHV4-4*02	IGHD2-21*02	IGHJ5*02	CARLPKHYIAEAVTW	11	IGKV1-5*03	IGKJ5*01	CQQYNSYPITF	9	5,13E+04	2,03E-04	3,96E-09	8,91E+04	2,91E-04	3,26E-09
V3	IGHV4-4*02	IGHD2-21*02	IGHJ5*02	CARLPKHYIAEAVTW	11	IGKV1-5*03	IGKJ5*01	CQQYNSYPITF	9						
V3	IGHV4-4*02	IGHD2-21*02	IGHJ1*01	CARLPKHYIAEAVTW	11	IGKV1-5*03	IGKJ5*01	CQQYNSYPITF	9						
V2	IGHV3-30*03	IGHD3-10*01	IGHJ5*02	CARDLYSGSGSNWATNRFDPW	17	IGKV3-15*01	IGKJ3*01	CQQYNSWPLTF	6						
V2	IGHV3-30*18	IGHD3-10*01	IGHJ5*02	CAKDLFSGSGSTWATNRLDPW	12	IGKV3-15*01	IGKJ3*01	CQQYNSWPLTF	6						
V3	IGHV2-5*10	IGHD1-1*01	IGHJ4*02	CARSVFPVLPFDFW	10	IGKV1-5*03	IGKJ1*01	CQEYSNYSGTF	8						
V3	IGHV2-5*10	IGHD1-1*01	IGHJ4*02	CARSVFPVLPFDFW	11	IGKV1-5*03	IGKJ1*01	CQEYSNYSGTF	8	5,58E+04	6,01E-05	1,08E-09	7,88E+04	1,09E-04	1,38E-09
V3	IGHV2-5*10	IGHD1-1*01	IGHJ4*02	CARSVFPVLPFDFW	11	IGKV1-5*03	IGKJ1*01	CQEYSNYSGTF	9	7,46E+04	4,29E-05	5,75E-10			
V2	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVVPAGVGFDYW	8	IGKV1-5*03	IGKJ2*03	CQQYNSYSSYDF	7						
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVVPAGVGFDYW	7	IGKV1-5*03	IGKJ2*03	CQQYNSYSSYDF	8	5,12E+05	5,60E-04	1,09E-09			
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVVPAGVGFDYW	7	IGKV1-5*03	IGKJ2*03	CQQYNSYSSYDF	7						
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVLPATFAFDFW	17	IGKV1-5*03	IGKJ1*01	CQHYDTYSWTF	7	4,36E+04	9,58E-05	2,20E-09	6,98E+04	1,68E-04	2,41E-09
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*03	CARTVLPATFAFDFW	16	IGKV1-5*03	IGKJ1*01	CQHYDTYSWTF	7						
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVLPATFAFDFW	16	IGKV1-5*03	IGKJ1*01	CQHYDTYSWTF	7						
V3	IGHV2-5*01	IGHD2-2*02	IGHJ4*02	CARTVLPATFAFDFW	16	IGKV1-5*03	IGKJ1*01	CQHYDTYSWTF	8						
V1	IGHV2-5*04	IGHD3-3*01	IGHJ4*02	CVHLPPYRISLFGEDLYRPTYFDFW	16	IGKV1D-33*01	IGKJ3*01	CQQYDDLPLTF	7						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	12	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	9						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGYVTVFGMILPRGWFDPW	11	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	7						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVKIFGMVLPRGWFDPW	10	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	10						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGYVTVFGMILPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8	1,41E+06	8,84E-05	6,26E-11			
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARVNGYVTVFGMILPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	7						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	7						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVMVFGMVLPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8						
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMILPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8	6,21E+04	6,87E-05	1,11E-09			
V1	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8	5,22E+04	2,10E-04	4,02E-09			
V2	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	10	5,19E+05	5,63E-05	1,08E-10	8,62E+05	1,40E-04	1,63E-10
V2	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	10						
V2	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVKIFGMVLPRGWFDPW	10	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	9	2,18E+05	2,25E-04	1,03E-09	5,63E+05	2,27E-04	4,03E-10
V3	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVPPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	7	2,13E+05	5,51E-05	2,58E-10	1,34E+05	2,47E-04	1,84E-09
V3	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARTNGNVTIFGMVLPRGWFDPW	8	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8						
V3	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8	2,23E+05	3,66E-05	1,64E-10	2,73E+05	1,77E-04	6,47E-10
V3	IGHV2-26*01	IGHD3-3*01	IGHJ5*02	CARINGNVTIFGMVLPRGWFDPW	9	IGKV1D-33*01	IGKJ4*01	CQQYDTLPLSF	8						
V1	IGHV2-26*01	IGHD6-13*01	IGHJ6*02	CARIQVVSAADKEGLYYYNAMDVW	14	IGKV1-5*03	IGKJ3*01	CQQYNSFSFTF	9						
V1	IGHV4-61*01	IGHD7-27*01	IGHJ4*02	CARVEWGSRRAFDSW	6	IGKV3-20*01	IGKJ3*01	CQQYGSSPLFTF	3	6,47E+05	1,72E-04	2,65E-10			
V2	IGHV4-61*01	IGHD7-27*01	IGHJ4*02	CARVEWGSRRAFDSW	6	IGKV3-20*01	IGKJ3*01	CQQYGSSPLFTF	3	6,47E+05	1,72E-04	2,65E-10	1,60E+06	5,60E-04	3,49E-10
V2	IGHV4-61*01	IGHD7-27*01	IGHJ4*02	CARVEWGSRRAFDSW	6	IGKV3-20*01	IGKJ3*01	CQQYGSSPLFTF	5						
V1	IGHV4-31*03	IGHD3-3*01	IGHJ5*02	CARRIAIFSVVLRSGWFDPW	13	IGKV1D-12*01	IGKJ5*01	CQQAYSFPITF	10	2,12E+04	1,13E-03	6,14E-08			
V3	IGHV4-31*03	IGHD2-15*01	IGHJ4*02	CARDMGSRVAAPLDYW	13	IGKV1-12*01	IGKJ4*01	CQQALSFPVTF	14	5,18E+04	1,23E-04	2,38E-09			
V3	IGHV4-31*03	IGHD2-15*01	IGHJ4*02	CARDMGSRVAAPLDYW	13	IGKV1-12*01	IGKJ4*01	CQQALSFPVTF	13						
V1	IGHV4-39*01	IGHD1-14*01	IGHJ3*02	CARPHVTWTSRTAFDIW	10	IGKV3-20*01	IGKJ5*01	CQQYGSSRSITF	0						
V1	IGHV4-39*01	ND	IGHJ5*02	CTNQMDNWFDPW	17	IGKV3-15*01	IGKJ2*01	CQQYDNWPPYTF	5						
V1	IGHV4-61*08	IGHD3-3*01	IGHJ4*02	CARRYDFWSGFLDYW	9	IGKV3-11*01	IGKJ5*01	CQTRNNWPLAF	4	1,41E+05	6,28E-04	4,46E-09			
V1	IGHV4-61*08	IGHD3-3*01	IGHJ4*02	CARRYDFWSGFLDYW	10	IGKV3-11*01	IGKJ5*01	CQTRNNWPLAF	6						
V2	IGHV6-1*01	IGHD1-26*01	IGHJ6*02	CARTSGGVEWLSSIYYGMDVW	5	IGKV1-12*02	IGKJ3*01	CQQANTFPFTF	10						
V2	IGHV6-1*01	IGHD1-26*01	IGHJ6*02	CARTSGGVEWLSSIYYGMDVW	5	IGKV1-12*02	IGKJ3*01	CQQANTFPFTF	10						
V2	IGHV6-1*01	IGHD1-26*01	IGHJ6*02	CARTSGGVEWLSSIYYGMDVW	4	IGKV1-12*02	IGKJ3*01	CQQANTFPFTF	10	7,78E+04	3,67E-05	4,72E-10			
V2	IGHV6-1*01	IGHD1-26*01	IGHJ6*02	CARTSGGVEWLSSIYYGMDVW	4	IGKV1-12*02	IGKJ3*01	CQQANTFPFTF	11						
V2	IGHV3-33*01	IGHD7-27*01	IGHJ4*02	CAAQSPFKGDHDYW	4	IGKV1-39*01	IGKJ2*02	CQQSYSSPPGF	4	1,09E+06	1,12E-04	1,03E-10			
V2	IGHV3-15*01	IGHD3-3*02	IGHJ6*02	CTTVENFWSEFYGMDVW	7	IGKV1-39*01	IGKJ4*01	CQQSYSTLTF	1						
V2	IGHV3-23*01	IGHD2-2*02	IGHJ4*02	CAKDLVIGQCTTTKCPRFFDSW	16	IGKV3-20*01	IGKJ4*01	CQLYGDPPPVTF	6						
V2	IGHV4-34*01	IGHD3-10*01	IGHJ3*02	CALDHYYGSGSYYNLPAFDIW	0	IGKV3-20*01	IGKJ1*01	CQQYGSSPLWTF	8	2,53E+06	1,06E-04	4,17E-11			
V2	IGHV3-15*01	ND	IGHJ3*02	CTTGGSLSMPMW	12	IGKV1-5*03	IGKJ2*03	CQHYSSYPYNF	3						
V2	IGHV4-59*01	IGHD3-10*01	IGHJ6*03	CARGEPDLYYYGSGSYSPRRVGDYYMDVW	3	IGKV1-39*01	IGKJ1*01	CQQSYSTPRTF	2	2,44E+05	7,07E-05	2,9E-10			
V2	IGHV4-39*01	IGHD2-2*02	IGHJ4*02	CARHGGYCSSASCYSGAFLTGYYFDYW	6	IGKV3D-20*01	IGKJ2*02	CQQYGSSPGTF	5						
V3	IGHV1-69*06	IGHD7-27*01	IGHJ6*02	CARDRLGTREYDWIFYGMEVW	18	IGKV1D-33*01	IGKJ4*01	CQQYDILPLTF	6	1,23E+05	1,12E-03	9,12E-09	8,19E+05	4,92E-03	6,01E-09
V3	IGHV1-69*06	IGHD7-27*01	IGHJ6*02	CARDRLGTREYDWIFYGMEVW	19	IGKV1-33*01	IGKJ4*01	CQQYDILPLTF	6						
V3	IGHV4-31*03	IGHD2-8*01	IGHJ4*02	CAREMVGHSAPADYW	12	IGKV1-39*01	IGKJ1*01	CQQTYKTPPTF	10						
V3	IGHV4-31*03	IGHD2-2*02	IGHJ4*03	CAREGYCSSIYCSFDYW	9	IGKV2-30*01	IGKJ1*01	CMQGTHWLPNV	7						
V3	IGHV3-23*01	IGHD6-6*01	IGHJ4*03	CAKSPEPIPARLAPGHFDYW	12	IGKV1-5*03	IGKJ1*01	CQQYNSYSPETF	8	6,12E+04	1,35E-04	2,21E-09	9,20E+04	2,59E-04	2,82E-09
V3	IGHV1-18*01	IGHD3-10*01	IGHJ4*02	CARTNNFGSGNYDAWNYFFDYW	19	IGKV1-12*01	IGKJ4*01	CQQGKSFPLSF	10						
V3	IGHV3-7*01	IGHD5-24*01	IGHJ4*02	CARWRWHQSEFDYW	7	IGKV1-39*01	IGKJ5*01	CQQNYSPPPITF	7						
V3	IGHV3-30*03	IGHD6-19*01	IGHJ4*02	CARGGVGVASKLSHW	21	IGKV1-5*03	IGKJ2*01	CQHYNRNPYTF	17						
V3	IGHV1-69*01	IGHD6-19*01	IGHJ4*02	CAMGDSSSKIEYW	8	IGKV2-30*01	IGKJ5*01	CMQGTHWPPITF	4						
V3	IGHV4-4*02	IGHD2-15*01	IGHJ3*02	CARVKGYCGGGRCHWGVSDIW	17	IGKV1D-33*01	IGKJ2*01	CQQYRNLPYAF	13	1,39E+04	8,08E-05	5,83E-09	9,42E+03	1,93E-04	2,05E-08
V3	IGHV3-74*01	ND	IGHJ4*02	CARDLGGIGSNW	10	IGKV2-28*01	IGKJ2*01	CMQALQTPYTF	7						
V3	IGHV3-48*03	IGHD3-9*01	IGHJ4*02	CVRERQDGTMLTGLFNYFDHW	8	IGKV1-39*01	IGKJ4*01	CQQSYSMPPLTF	4						
V3	IGHV1-18*01	ND	IGHJ4*02	CAREYGDYKFDYW	7	IGKV1-12*01	IGKJ4*01	CQQADSFPLTF	4	6,80E+04	3,65E-05	5,37E-10			
V3	IGHV3-21*01	ND	IGHJ5*02	CSRDEVNRPHYIW	14	IGKV1-5*03	IGKJ1*01	CQQYNSYSWTF	3						
V3	IGHV3-53*01	IGHD1-7*01	IGHJ4*02	CGNYDWNYGGVEFW	15	IGKV1-39*01	IGKJ2*01	CQQSYSSPPYTF	14						

Supplementary Table 1. IgH and Igκ chain gene sequence information and antibody binding affinity. ND indicates not determined. One member of each clonotype is indicated with the number of clonal relatives with various degrees of somatic mutations. a-b, Antibodies from tetanus specific IgG or IgA B cells from donor TT1 and TT2. Clonotypes are separated by alternating grey coloring.

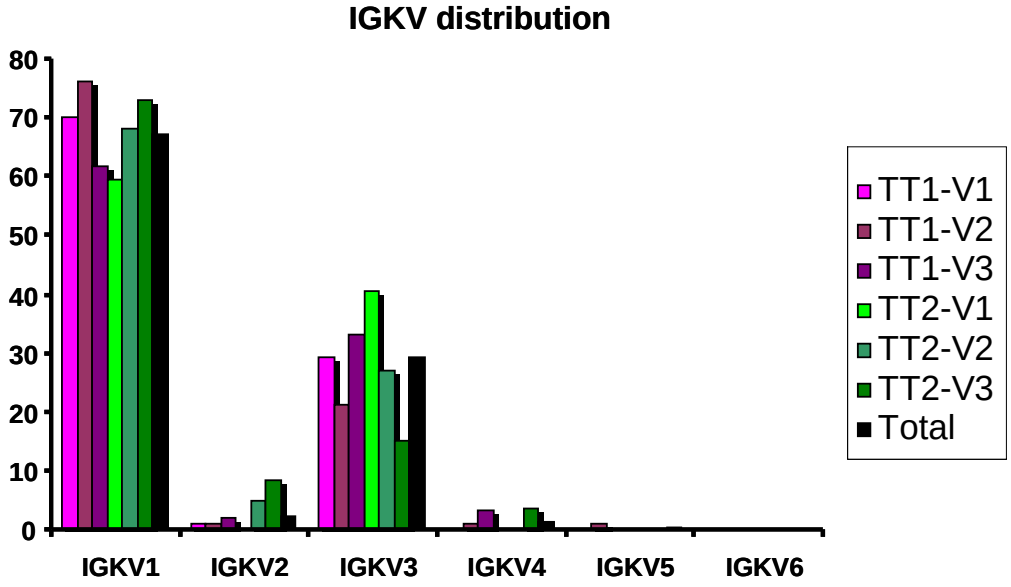
Supplementary Figure 1a. VH gene segment usage in the six antibody repertoires.



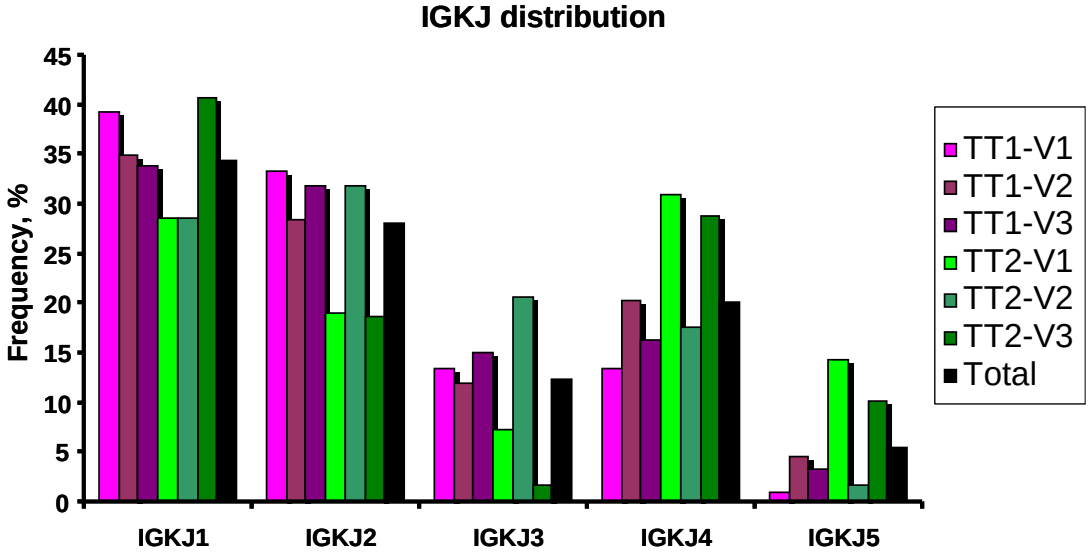
Supplementary Figure 1b. JH gene segment usage in the six antibody repertoires.



Supplementary Figure 1c. Vκ gene segment usage in the six antibody repertoires.



Supplementary Figure 1d. Jκ gene segment usage in the six antibody repertoires.



Supplementary Figure 1. V and J gene segment usage in the six antibody repertoires. Gene segment usage for donor TT1 and TT2 are indicated in purple and green colors, respectively. The total gene distribution for both donors is indicated in black.

Paper 3

The role of genetic diversification mechanisms in antigen-induced antibody repertoires

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Abstract

The humoral immunes system relies on a vast diversity of antibodies to combat foreign invaders. We have previously employed the Symplex technology for isolating antibody repertoires from two hyperimmune donors upon three consecutive immunizations with tetanus toxoid. A total of 594 unique antibodies with cognate heavy and light chain pairing were isolated from these donors. The isolated repertoires were diverse in terms of number of hypermutations and there was no change in either repertoire size or the number of somatic mutations although the repertoires appeared dynamic with an influx/efflux of clonotypes. Further, we reported affinities for a total of 156 antibodies from these repertoires which reached an affinity ceiling after the second immunization. Here we set out to characterize the genetic development and diversification of these large late repertoires and compare these to a repertoire from one tetanus naïve donor after receiving the first vaccination. We isolated 11 tetanus specific antibodies from this primary repertoire. The consecutive repertoires from the two hyperimmune donors were diverse in terms of gene segment usage which did not change after several immunizations. Further, the usages were similar to that of the primary repertoire. We did not identify any insertions or cases of receptor editing indicating that these processes are not major contributors to diversification. Deletions were observed in 6% of the isolated clonotypes. Surprisingly, the primary repertoire isolated on day 6 after immunization consisted of antibodies with relatively high numbers of somatic hypermutations averaging 15 per antibody suggesting that somatic hypermutation occurs very fast after the first encounter with antigen. The affinity of one antibody from the primary repertoire was determined to be 0.26nM indicating that the primary tetanus repertoire includes high affinity binders. All the affinities were correlated to the number of hypermutations of each antibody. There was no correlation between on-rates, off-rates or affinities and the number of hypermutations even for antibodies of common clonal origin. However, off-rates were improved by the introduction of hypermutations to germline antibodies. Our results demonstrate that tetanus specific repertoires are highly diverse even after a single vaccination and they keep the level of diversification even after many

antigenic challenges. Further our results indicate that the quality of the primary antibody repertoire against a physiologically relevant antigen is better than predicted from hapten studies.

Introduction

The immune system is known to generate a diverse repertoire of high affinity antibodies when exposed to foreign substances. The massive diversity of naïve antibody repertoires is generated by recombination of immunoglobulin V, (D) and J gene segments during the early stages of B cell development (39). Later upon encounter with antigen, B cell receptors undergo somatic hypermutation (SHM) in germinal centers (15) creating further antibody diversity. During the course of an antigen-specific immune response, the affinity of serum antibodies has been observed to increase with time (7;35). Such a maturation results from specific alterations of the structure of the antibody molecules. Studies on specific hapten responses have led to the conclusion that the introduction of somatic hypermutations to germline antibodies increases the affinity of the antibody (36). In addition to the structural diversity introduced by the recombination of different gene segments and the generation of further diversity by somatic hypermutations, entire codons can be inserted or deleted in rearranged antibody genes. These often result in the generation of new combinations of canonical loop structures or entirely new loops creating an expanded antibody structure space (10). About 1-10% of normal B cells have been reported to carry insertions or deletions in their hypermutated antibody-encoding genes (10;12;23;30;44).

Selection during affinity maturation ensures that the immune system creates a diverse repertoire of high quality specific antibodies in response to virtually any antigen. In recent years, newly developed antibody isolation techniques (16;20;21;27;38;40;43;45;46) have helped reveal the genetic makeup of specific antibody repertoires and characterize the diversity of these. However, the knowledge is still limited on genetic diversification and development of specific antibody repertoires.

We have recently used the Symplex™ technology (22) for determining the biological limits for antigen-driven affinity maturation by isolating comprehensive antibody repertoires from two adult volunteers after receiving three consecutive booster vaccinations with tetanus toxoid separated by 4.5 and 1.5 years (26). Here we set out to characterize the development and diversification of these large repertoires consisting of 594 antibodies combined with a repertoire isolated from an adult tetanus-naïve donor 6 days after the very first tetanus vaccination. The repertoires from the two previously described hyperimmune donors comprised 382 unique antibodies from donor TT1 (120, 108 and 154 antibodies from the first, second and third repertoires, respectively) and 212 antibodies (84, 69 and 59 antibodies from the first, second and third repertoires, respectively) from donor TT2. A primary repertoire of 11

tetanus specific antibodies was isolated from the naïve donor, TT3. Hence, a total of 605 unique antibodies with cognate heavy and light chain pairing were isolated from these three donors. Table 1 shows all repertoire numbers.

The repertoires were analyzed genetically by cluster analysis and assignment of gene segment usage and number of hypermutations. Representative subsets of these repertoires were expressed and purified for kinetic measurements in order to determine the role of hypermutation on binding in developing antibody repertories.

Tetanus toxoid was chosen as antigen for these studies because it is one of the most potent vaccines in clinical use and it is safe. Further, it is a structurally complex antigen ensuring that the studied antibody repertoires are not restricted by the antigen. We anticipate that our tetanus specific results on developing antibody repertoires and diversification mechanisms can be transferred to a more general understanding of specific antibody repertoires.

Results

Previously, 382 unique antibodies were isolated from donor TT1 (120, 108 and 154 antibodies from the first, second and third repertoires, respectively) while 212 antibodies (84, 69 and 59 antibodies from the first, second and third repertoires, respectively) were isolated from donor TT2. We have now further isolated 11 antibodies from the naïve donor, TT3. Table 1 summarizes all repertoire numbers.

Cluster analysis of specific antibody repertoires from hyperimmune donors

In order to determine the diversity and development of the three consecutive repertoires from each hyperimmune donor, we have previously conducted a cluster analysis and assigned sequences to clonotypes by the clustering. Unique V genes were identified based on the deduced amino acid sequences from DNA sequencing. Antibodies were assigned the same clonotype if they shared V_H, J_H, V_K, and J_K family segments, heavy chain CDR3 lengths and a consensus sequence in CDR3 for both heavy and light chains. 33 and 43 clusters of high sequence homology (clonotypes) were identified from donor TT1 and TT2, respectively. From donor TT1, 26, 59 and 54 singletons were isolated from the first, second and third repertoires, respectively, while the numbers for donor TT2 were 46, 45 and 41.

Figure 1 demonstrates the distribution of antibodies previously analyzed to be in different clusters for the 3 repertoires from each donor. Most of the clusters from donor TT1 were represented by antibodies from either two or all three repertoires. No new clusters were formed in the third repertoire although new singletons arrived. For donor TT2, the picture was a bit different. Here only the four larger clusters were represented by antibodies from all three repertoires and most clusters were only found in one repertoire. Additionally, new clusters were isolated in all three repertoires. In total, 260 and 81 antibodies were represented in at least two repertoires from donor TT1 and TT2, respectively. This corresponds to 24 and 14 clonotypes for donor TT1 and TT2, respectively. The distributions demonstrate that there was a higher likelihood of finding new members of the larger older clusters when isolating consecutive repertoires.

These findings supplement our earlier findings (26) that mature tetanus specific antibody responses are similar in size but dynamic regarding the clonotypes represented in each repertoire after consecutive immunizations.

The early specific repertoire

The diversification of early antibody responses in humans is not well described. In order to compare the diversification and development of the hyperimmune repertoires to an early repertoire, the Symplex technology was employed for the isolation of a primary antibody repertoire from an adult tetanus-naïve donor (TT3) 6 days after receiving the tetanus vaccine for the very first time.

A rather small number of 15 positive hits were obtained of which 11 were unique sequences of functional antibodies. A cluster analysis revealed that there were two clusters of similar antibodies with seemingly common clonal origin consisting of two antibodies each and 7 singleton antibodies yielding a total of 9 clonotypes, see table 2. These numbers were used to predict a maximum likelihood estimate on the actual repertoire size (4;27). The repertoire was most likely composed of 24 clonotypes (maximum likelihood estimate) at the time of isolation. The confidence interval (CI) was large with a 95% CI at 71 clonotypes and hypermutated variants thereof. This size is, however, within the same range of under 100 clonotypes as repertoire sizes we have previously estimated for hyperimmune tetanus repertoires (26).

Distribution of variable gene segments in human anti-TT antibody repertoires

Major structural restrictions of antibody repertoires can be identified by a genetic analysis of the variable region gene segment usage. Previously, we have therefore analyzed the distributions of V gene segments and J gene segments for the V_H and V_K genes in the first repertoires from donor TT1 and TT2 (27). Here, we included the second and third repertoires from these donors and the primary repertoire from donor TT3 which were all analyzed and compared to previously described naive peripheral IgM^+ B cell repertoires of between 206-350 antibody clones (8;11) (figure 2). In accordance with earlier observations, the TT repertoires of all three donors showed extensive diversity in their V gene segment usage. The five most prevalent H chain V gene segment families were found with only minor differences relative to the naive repertoire (figure 2A). The H chain J gene segment usage also followed the naive repertoire except for the J_H5 gene segments, which were less frequently used (figure 2B). The distribution of kappa-chain V gene families followed the naïve repertoire dominated by the V_K1 and V_K3 families, although the V_K2 gene family was less prevalent in the TT repertoires (figure 2C). Similarly, kappa-chain J gene segment usage showed a similar distribution between the repertoires (figure 2D). Thus, all the TT repertoires – even the primary – showed extensive diversity without any

major biases in V and J gene segment usage. Further, the usage of V_H, J_H, V_K, and J_K segments did not seem to change in the three consecutive repertoires from each donor. Rather the usage of each segment was relatively constant and similar for both hyperimmune donors. The level of diversification in the primary repertoire seemed similar to the repertoires from the two hyperimmune donors. The fact that the primary repertoire consisted of at least 9 clonotypes using several different gene segments indicates that the primary tetanus specific repertoire is not restricted to few clonotypes.

Somatic hypermutations

In early hapten studies on development of antibody repertoires, Berek et al. reported that antibodies of primary repertoires displayed few or no somatic hypermutations on day 7 after immunization (7). In the later primary responses the numbers increased to up to 5 or 6 amino acid replacements per antibody and they were further increased upon several antigenic challenges. Therefore we analyzed the numbers of somatic hypermutations (SHM) for characterization of the maturity level and development of antibody repertoires. As reported previously (26), the number of amino acid (aa) SHM per antibody varied between 0 and 40 for hyperimmune responses with 50% of the antibodies having between 16 and 25 SHM. As we then demonstrated, the numbers of hypermutations in these mature and saturated antibody repertoires were rather constant after several immunizations but differed slightly between individuals; SHM averaged 23 and 18 for donor TT1 and TT2, respectively. We also showed that the hypermutation data generally followed normal distributions for V_H, V_K, and V_H+V_K, although there was a slight bias for zero hypermutations (data not shown).

SHM did not seem to continue excessively in matured antigen-specific cells; on two occasions, we found antibodies with identical amino acid sequence as found in earlier repertoires from the same donor indicating that some memory B cells escape new somatic hypermutation rounds upon the next encounter with antigen. Additionally, the mature antibody repertoires from donor TT1 included 6 tetanus specific non-hypermutated antibodies (antibodies in germline configuration). In repertoires from both hyperimmune donors, new members of old clusters generally did not have more hypermutations, many even had less than older members (data not shown).

Figure 3 shows the number somatic hypermutations in the heavy and light chain for each of the 11 antibodies from the early antibody repertoire. The numbers of SHM spanned 6-22 amino acid replacements with an average of 15 which was surprisingly high at this early stage after the first

immunization. Also, it was higher than expected compared to the slightly higher numbers reported earlier in the hyperimmune repertoires. The relatively large numbers of hypermutations found in all primary antibodies indicates that somatic hypermutations occur fast after the first tetanus challenge.

Insertions and deletions

Insertions and deletions of entire codons have been suggested to contribute to diversification of antibody repertoires (23). We therefore analyzed whether our repertoires displayed insertions and deletions.

Amino acid deletions had occurred in 6.4% of the clonotypes isolated based on analysis of V segments only, see figure 4. All deletions involved 1-5 aa with a small majority found in heavy chains with a frequency of 3:2 compared to light chains. No insertions were identified. Almost all deletions were found close to CDR1s or CDR2s in both heavy and light chains. Only a single antibody had a three aa deletion in framework 3 at positions 88-90 of its light chain. Insertions and deletions in the CDR3 regions were not included in the analysis.

The low number of observed deletions and the complete absence of identified insertions suggest that these mechanisms are not important in shaping tetanus specific antibody repertoires.

The influence of hypermutation on binding

The increase in the number of somatic hypermutations during antibody repertoire development has been connected with an increase in affinities (7). As previously reported, we have determined affinities and on- and off-rates for 156 antibodies from the TT1 and TT2 repertoires (26). Here we also determined the binding kinetics and affinity of an antibody from the primary repertoire from TT3. Remarkably, this very early antibody displayed a high affinity of 0.26nM.

Previously, we have plotted the number of hypermutations against the number of SHM in the first repertoires from the hyperimmune donors to test whether there was a correlation of number of SHM of each antibody and its binding kinetics (27). Here we included the affinities from the other two repertoires from the hyperimmune donors as well as the affinity from the primary repertoire, figure 5. Based on the 157 unique tetanus specific antibodies, we did not observe a correlation between the number of hypermutations for each antibody and its affinity to tetanus toxoid (see figure 5A). Neither did we observe a correlation between the number of SHM and on-rates (figure 5B) or off-rates (figure 5C). Thus high numbers of hypermutations did not seem to correlate with either high or low affinity confirming our previous findings (27). In that study we also tested whether this was valid for a limited number of clonally related antibodies by plotting the on- and off-rates. Here we included 37 antibodies from the 6 larger clusters from TT1 and TT2 to test whether this also applied to related antibodies from different repertoires (figure 6). The number of hypermutations was displayed for each antibody. Just as in our previous study (27), there was an absence of a relationship between the number of hypermutation and binding kinetics or affinity for these clonally related antibodies. Further, there did not seem to be a one-directional development in either on-, off-rates or affinities for clonally related antibodies found in later repertoires than their cluster sisters from earlier repertoires. Hence this plot confirmed the lack of correlation observed in figure 5.

In order to examine whether the lack of correlation between the number of SHM and binding kinetics was applicable to hypermutated antibodies only, we plotted the binding kinetics of two antibodies in germline configuration (non-hypermutated) isolated from donor TT1 (from repertoires 1 and 2), see figure 7. Also, we plotted the kinetics of two antibodies in deduced germline configuration described earlier (27) and hypermutated variants of these isolated from donor TT1 in all three consecutive repertoires. The kinetics for all of the plotted antibodies are summarized in table 3. We did not isolate any hypermutated variants of the two germline antibodies isolated directly from one of the donors. The number of amino acid hypermutations for heavy and light chains was displayed for each of the antibodies. Both of the germline antibodies isolated from one of the donors were of intermediate to low affinity comparable to one of the antibodies in deduced germline configuration. However, both displayed much slower on-rates and slower off-rates. We did not observe a strict relationship between the number of SHM and on-rates or affinity. However, all of the hypermutated variants of the two antibodies in deduced germline configuration displayed improved off-rates. The on-rates changed in

both positive and negative directions or were neutral resulting in hypermutated antibodies with both better, worse and equal affinity to their supposed ancestor.

Discussion

Combining the present and earlier studies (26;27), we have isolated a total of 605 unique tetanus specific antibody sequences with cognate pairing of heavy and light chains using the Symplex technology. The majority of the sequences were derived from three consecutive repertoires isolated from each of two hyperimmune donors (26) whereas the last 11 sequences were from a tetanus-naïve donor who received the tetanus vaccine for the first time. We have analyzed these primary and late antibody repertoires to examine the development of specific antibody repertoires at early and late stages.

The number of hypermutations found in the naïve donor was higher than expected only six days after the very first tetanus vaccination especially compared to what has been observed in primary responses against hapten (7). The total numbers for heavy and light chains combined are within the same magnitude as observed for the hyperimmune donors but at the lower end. These numbers also correlate well to numbers reported by others (21;33;37;45;46). One could suspect that the donor had encountered tetanus previously by natural exposure because of these relatively high numbers of SHM without developing disease which of course would have been recognized. However, the fact that so few tetanus specific antibodies were isolated compared to the repertoires of the hyperimmune donors is a strong indication that the donor was truly naïve at the time of vaccination. Kodo et al. (19) have observed a low number of specific antibody producing cells after a primary immunization with tetanus in humans compared to secondary or later vaccinations. Additionally, Andreas Radbruch has reported that the day when antigen-specific cells peak in the blood after vaccination varies between day 5 and 9 depending on the donor (28).

The size of the early specific antibody repertoire is small but the fact that we find two clusters of clonally related antibodies among only 11 antibodies suggests that we have a good sampling of the repertoire suggesting that the actual repertoire was small at this stage. Again, this is indicative of a truly primary response which was also confirmed by a very low frequency of positive hits compared to the hyperimmune repertoires. The relatively large numbers of hypermutations found in all antibodies indicates that somatic hypermutations occur very early after the first encounter with antigen. The fact that the primary repertoire consisted of at least 9 clonotypes using several different gene segments indicates that the primary tetanus specific repertoire is not restricted to few clonotypes or gene

segments but it is diverse. This diversity at this early stage is reflected in the diversity observed in later responses.

From the second to the third vaccination of the two hyperimmune donors there was no increase in repertoire size, somatic mutations, or change in the distribution of association rate, half life or affinity (26). Further, the gene segment usage did not change after several immunizations and did not seem to deviate from previously described naive peripheral IgM^+ B cell repertoires (8;11) or the early repertoire investigated. These distributions seemed relatively constant after several immunizations indicating that the anti-tetanus repertoires were not skewed in a certain direction with more antigenic challenges. The early repertoire reflected the gene segment usages of both the naïve B cells and the hyperimmune repertoires. This indicated that the repertoires did not develop further genetically but instead kept reflecting the natural diversity of naïve repertoires. The relatively constant number of clusters and singletons observed in each repertoire for each donor was indicative of a rather constant sized repertoire consisting of a "base" and an influx/efflux of antibody clones. The fact that so few of the clusters from TT2 were represented in more than 1 repertoire compared to the TT1 repertoires could be due to a significantly smaller sample size.

When analyzing the sequences for insertions and deletions, it was apparent that most deletions were found close to CDR1s or CDR2s in both heavy and light chains while we did not identify any insertions. The proximity to CDRs supports findings by de Wildt et al. (10) that the processes often result in the generation of new combinations of canonical loop structures or entirely new loops which are then likely to contact the antigen directly. The fact that they are mostly found in conjunction with CDRs is probably because this is the only positions where larger molecular alterations can be tolerated without disrupting the immunoglobulin structure. Our results demonstrate that deletions are indeed found in mature specific antibody repertoires against tetanus. However, the relatively small fraction of the repertoires in which deletions were observed (6%) and the lack of identified insertions suggest that they are not a dominant means of diversification.

We did not observe any instances of receptor editing in our repertoires. Yet we cannot rule out that the process had occurred. Receptor editing does, however, not seem to be a dominating diversification mechanism of developing antibody repertoires as has been proposed (24;25;29) although we may underestimate the extent of the process.

Early immunologic studies on hapten responses have concluded that the introduction of hypermutations and clonal selection leads to a gradual increase in affinity of specific serum antibodies after repeated exposures (7;36). Further, Sagawa et al. have observed a positive correlation between on-rates and SHM in a hapten response (32). We were not been able to establish a relationship between on-rates or affinities against the total number of hypermutations. However, we did observe an improvement in off-rates of all hypermutated variants of the two antibodies in deduced germline configuration. Further, we observed an overall improvement in affinity (and on- and off-rates) upon consecutive immunizations of the same donors within relatively short time intervals (1.5-4.5 years) (26). When only looking at the first repertoires from the two hyperimmune donors, we suggested that overall a higher average number of hypermutations in one of the donors correlated with this donor having overall higher affinities than the other donor (27). However, when looking at all three repertoires from each donor, it is apparent that the average number of SHM did not change for any of the donors (although it differed between the donors) but both donors improved the overall affinities after the first repertoire and kept the level after the second. This indicates that each hypermutation has truly stochastic effects but combined with others and selection they aid in improving the binding kinetics of a specific antibody repertoire. This is consistent with the fact that the one antibody from the primary repertoire with a determined affinity is in the high end of the affinity span observed for the other repertoires although it has only 13 hypermutations. Further, we have previously found that non-hypermutated can be of high affinity as well (27).

Berek and Milstein (6) have argued that antigenic stimulation of memory B cells leads to further processes of hypermutation and selection, probably in germinal centers. The fact that we found antibodies with identical aa sequence as some found in earlier repertoires indicates that some B cells escape the SHM machinery upon secondary encounters with antigen. Further, many of the antibodies found in our later repertoires were equally or less hypermutated than their “cluster sisters”. Hence, our data indicates that not all memory cells are hypermutated upon second or later encounters with antigen.

We have found that both the hyperimmune repertoires and the small primary repertoire are relatively diverse with up to the order of 100 clonotypes which indicates that tetanus specific repertoires are generally diverse compared to some specific repertoires e.g. against influenza (vaccine) (45), Rhesus D (2), rotavirus (18) and Haemophilus influenzae type B (Hib) (1;3;14;34). However, other specific repertoires have been demonstrated to be as diverse as our tetanus repertoires. These

include HIV (33), Vaccinia (5) and vesicular stomatitis virus (VSV) (17;31). The diversity we observe is supported by early findings by Volk et al. who have observed specific antibodies against up to ~24 different epitopes against tetanus toxin (41).

Germinal center reactions have been shown to be pauciclonal, founded, on average, by three or fewer B lymphocytes (15). Hence, memory cells from former tetanus responses may not be represented in all new germinal centers that are formed following a challenge. Thus all new TT binding clones may not have to compete against the very best clones from former responses. This could partly explain the relatively large influx of new clones and why there are still low affinity clones in the later responses. The theory is supported by the work by Dal Porto et al. (9) in which they argue that affinity maturation during the primary GC reaction appears to be local, i.e. competition between B cells take place within but not between GCs. The conclusion to these observations is that each GC defines a local affinity optimum. Additionally, Vora & Manser have suggested that the pauciclonality of each GC results in antibody clones competing almost exclusively against their own hypermutated variants (42). Hereby, clonal selection is based on clones with better affinities than their own hypermutated "sisters" rather than affinities that are superior to all other antigen specific clones. This theory could explain the large clonal influx into hypermutated repertoires and the large span we observe in affinities. Further, it is consistent with the fact that we can correlate hypermutations to better off-rates for hypermutated variants of germline antibodies. However, the latter may suggest that affinity maturation within GCs is more dependent on off-rates than affinities.

In summary, our previous studies have demonstrated that the genetic composition of tetanus specific antibody repertoires is highly diverse and similar to that of naïve B cells. Here we have shown that the diversity is evident after a single vaccination and does not change with many antigenic challenges. Also, we demonstrate that somatic hypermutations occur early after the first antigen encounter. As early as day 6 after the first vaccination, the level of mutations was surprisingly high with an average of 15 amino acid mutations per antibody. The hyperimmune repertoires, we have previously described, have slightly increased numbers of hypermutations compared to the primary repertoire and as reported earlier, they finally reach a ceiling. In our large data sets, we did not observe any instances of receptor editing suggesting that this is not an important mechanism for antibody

diversification. Further, we identified few antibodies with deletions and none with insertions – again indicative of a minor contribution by these processes to antibody diversification.

The number of hypermutations for each antibody did not correlate to affinity, or on- rates confirming our previously reported results. However, off-rates were improved when mutations were introduced to germline antibodies. Based on these observations we conclude that somatic hypermutations are important for improvements in off-rates of germline antibodies. This is supported by our finding that somatic hypermutations occur very fast in the primary tetanus repertoire.

Our findings indicate that the quality of the primary antibody repertoire against physiologically relevant antigens is better than predicted from hapten studies. It may therefore be of use in the development of future antibody therapeutics.

Table 1

Results of Symplex™ repertoire cloning from 3 repertoires from each of the two hyperimmune donors and one primary repertoire from a tetanus naïve donor. Repertoire size was estimated by gel electrophoresis of a representative set of single cell RT-PCR samples (from 10-20% of the total). Unique V genes were identified based on the deduced amino acid sequences from DNA sequencing. Antibodies were assigned the same clonotype if they shared V_H, J_H, V_K, and J_K family segments, heavy chain CDR3 lengths and a consensus sequence in CDR3 for both heavy and light chains.

Donor	Boost no.	Estimated Repertoire Size (unspecific)	No of bacterial clones screened	No. of TT-positive clones	No of sequenced clones	No of unique VH-VK amino acid sequences	Estimated number of clonotypes
TT1	V1	400	3600	338	169	120	30[a]
TT1	V2	1700	2100	427	155	108	50
TT1	V3	2400	2600	372	176	154	67
TT2	V1	400	3400	187	102	84	42[a]
TT2	V2	1600	3500	95	82	69	36
TT2	V3	1400	3600	104	83	59	29
TT3	V1	1400	2100	15	11	11	9
Total		**9300**	**20900**	**1538**	**778**	**605**	**204**[b]

[a]: The estimated numbers of clonotypes from Poulsen et al. (27) (TT1-V1 and TT2-V1) were adjusted from 29 and 40 to 30 and 42, respectively, due to a slightly stricter clonotype definition.

[b]The total estimated number of clonotypes is lower than the sum of numbers for all repertoires since many clonotypes were shared between repertoires for each donor.

Table 2

Overview of 11 antibodies from a primary anti-tetanus repertoire isolated from a naïve donor after receiving a tetanus vaccination. Each clonotype is marked by its own color.

Clone	Affinity	Total no. Of SHM	HC SHM	LC SHM	VH	D	JH	HCDR3	VK	JK	LCDR3
_350j11	**$2{,}6x10^{-10}$**	13	9	4	IGHV3-11*03	IGHD6-13*01	IGHJ6*02	CVRGPEDSSRWYAPSYYYYYYGMDVW	IGKV1-39*01	IGKJ4*01	CQQTYSPPLTF
_355G12	**-**	12	8	4	IGHV3-11*03	IGHD6-13*01	IGHJ6*02	CVRGPEDSSRWYAPSYYYYYYGMDVW	IGKV1-39*01	IGKJ4*01	CQQTYSPPLTF
_354B23	**-**	22	14	8	IGHV3-23*01	IGHD2-15*01	IGHJ4*02	CAKADAETCSGRRCYAFDSW	IGKV3-11*01	IGKJ5*01	CQQHSNLITV
_355J06	**-**	17	13	4	IGHV3-23*01	IGHD2-15*01	IGHJ4*02	CAKADAETCSGRRCYAFDSW	IGKV3-11*01	IGKJ5*01	CQQHSNLITF
_355K12	-	21	11	10	IGHV3-15*04	IGHD6-19*01	IGHJ5*02	CTHYTSGWFW	IGKV3-20*01	IGKJ2*01	CQQYSTSVPYTF
_355H12	-	18	14	4	IGHV1-69*01	IGHD2-21*02	IGHJ4*02	CARKGSGADDYFDHW	IGKV3-11*01	IGKJ3*01	CQQRSSWPMITF
_355I08	-	14	10	4	IGHV1-69*01	IGHD3-16*02	IGHJ4*02	CASRRALSTTEVADYW	IGKV3-20*01	IGKJ1*01	CQQYGSSPMTF
_355j05	-	6	5	1	IGHV3-11*03	IGHD3-10*01	IGHJ4*02	CASIGMGRGVDYW	IGKV2-30*01	IGKJ1*01	CMQGTHWPWTF
_355O23	-	13	10	3	IGHV3-33*01	IGHD2-2*01	IGHJ1*01	CARSPLVAAAVDYW	IGKV3-20*01	IGKJ4*01	CQQYGSSPLTF
_355N15	-	22	12	10	IGHV4-34*01	IGHD3-10*02	IGHJ4*02	CVRVPPIIAAADNW	IGKV1-9*01	IGKJ3*01	CQHLNSYPLRFTF
_355B16	-	9	4	5	IGHV5-51*01	IGHD3-9*01	IGHJ4*02	CIAARANHEQRYLDWFADYW	IGKV1-5*03	IGKJ1*01	CQQYSFYGTF

Table 3
Kinetics and affinity constants of Abs with deduced germline variable region genes and their hypermutated variants and the influence of hypermutations on antibody binding.

Boost no.	Clone	On-Rate (M^{-1} s^{-1})	Off-Rate (s^{-1})	$t_{1/2}$ (min^{-1})	Affinity (M)	No. of hypermutations (VH + VK)
-	Germline VH1-69-JH6 VK1-27-JK3	3.0×10^{6}	3.3×10^{-4}	35	1.1×10^{-10}	0 + 0
1	60D05 (VH1-69-JH6 VK1-27-JK3)	6.4×10^{6}	1.7×10^{-5}	670	2.7×10^{-12}	13 + 6
1	62D09 (VH1-69-JH6 VK1-27-JK3)	2.7×10^{6}	5.6×10^{-5}	210	2.1×10^{-11}	10 + 6
1	63B12 (VH1-69-JH6 VK1-27-JK3)	4.6×10^{6}	7.4×10^{-5}	160	1.6×10^{-11}	10 + 4
2	338H08 (VH1-69-JH6 VK1-27-JK3)	2.1×10^{5}	1.9×10^{-5}	599	9.0×10^{-11}	12 + 6
2	339H12 (VH1-69-JH6 VK1-27-JK3)	6.8×10^{4}	6.0×10^{-5}	194	8.7×10^{-10}	13 + 6
3	528H18 (VH1-69-JH6 VK1-27-JK3)	4.0×10^{6}	7.7×10^{-5}	150	1.9×10^{-11}	10 + 6
3	528K04 (VH1-69-JH6 VK1-27-JK3)	7.0×10^{5}	5.1×10^{-5}	227	6.7×10^{-11}	9 + 5
-	Germline VH3-21-JH6 VK1-39-JK1	6.8×10^{5}	1.7×10^{-1}	0.1	2.5×10^{-7}	0 + 0
1	63D10 (VH3-21-JH6 VK1-39-JK1)	1.0×10^{5}	4.2×10^{-4}	28	4.0×10^{-9}	14 + 13
1	60A02 (VH3-21-JH3 VK1-27-JK2)	9.1×10^{3}	1.3×10^{-4}	90	1.4×10^{-8}	0 + 0
2	340N16 (VH3-30-JH3 VK1-5-JK2)	1.8×10^{2}	3.0×10^{-5}	381	1.7×10^{-7}	0 + 0

Materials and Methods

Donors and vaccination

Previously, two healthy donors were boosted with the tetanus vaccine (Statens Serum Institut, Denmark) containing tetanus toxoid in three consecutive boosts under informed consent (26;27). Now a tetanus naïve donor was recruited in addition and vaccinated for the first time with the tetanus vaccine (Statens Serum Institut, Denmark) also under informed consent. The project was approved by the regional ethical committee in Copenhagen, Denmark. Blood was harvested in anticoagulant 6 days after vaccination.

Antibody repertoires from the two hyperimmune donors

The three consecutive tetanus specific antibody repertoires from each of the two hyperimmune donors were isolated, screened and characterized as described previously (26).

Isolation of plasma blasts and cell sorting

Peripheral blood mononuclear cells (PBMC) from the previously tetanus-naïve donor were purified by density centrifugation using Lymphoprep (Axis-Shield PoC AS) and stained with anti-CD19-FITC (Becton Dickinson) in MACS buffer (phosphate buffered saline (PBS), pH 7.2, 0.5% (w/v) bovine serum albumin (BSA), 2mM EDTA), washed and labeled with anti-FITC conjugated magnetic beads in order to enrich for B cells on a MACS LS column (Miltenyi Biotec) according to the manufacturer's recommendations. The repertoire was sorted as previously described (27;27). Shortly, the CD19-enriched fraction was resuspended in FACS buffer (PBS, pH 7.2, 2% fetal calf serum (FCS)) and incubated with anti-CD19-FITC (Becton Dickinson), anti-CD38-APC (Becton Dickinson), anti-lambda-PE (Becton Dickinson), and anti-CD45-PerCP (Becton Dickinson) for 20 minutes at 4°C in the dark, washed and resuspended in FACS buffer and stained for viability with propidium iodide (PI). Viable cells were sorted based on the following expression profile: $CD38^{high}/CD19^{int}/CD45^{int}/lambda^{neg}$. Single cell sorting was performed on a FACSAria cell-sorting system (Becton Dickinson) where single cells where sorted directly into 384 well PCR plates (Applied Biosystems).

FACS analysis was performed using FACSDiva software. 97-99% purity was detected on the single cell sorting.

Plasma blasts were sorted directly into wells containing all reagents necessary for the Symplex PCR except for reverse transcriptase, DNA polymerase and dNTPs as described previously (27) and stored at -80°C for later processing.

Single cell RT-PCR and PCR of antibody variable region genes

V_H and LC_κ genes were amplified and linked through primer overlap extension as described previously (22). Briefly, V_H and LC_κ from each cell were amplified in a one-step RT-PCR reaction using a cocktail of sense primers specific for the leader regions and antisense primers to the Cγ constant regions for heavy chains and Cκ for the light chain. One microliter from each RT-PCR reaction was transferred to new plates containing reagents for a nested PCR and V_H and LC_κ genes were linked and amplified in separate PCR reactions using nested primers with overlap extensions and containing restriction sites at the ends of the heavy chain variable regions and the full κ light chains as previously described (22).

Cloning and Screening

Cloning and screening were performed as described previously (26;27). Linked antibody gene fragments (VH-LCκ) were pooled and cloned into expression vectors using the PCR introduced restriction sites as described previously (22). For the first two repertoires from each donor, linked antibody genes were cloned into a bacterial or mammal Fab expression vector (22;26) and the gene linker was replaced by a bidirectional bacterial/mammal promoter fragment (lac-tac/2xCMV) by sub-cloning. The vectors from the naïve repertoire and the first two hyperimmune repertoires were transformed into bacteria (*E.coli*, TOP10) by electroporation and transformants were selected on 2xYT or LB broth agar containing 100μg/ml of carbenicillin. Single *E.coli* colonies were picked into 96 or 384 well plates. Fabs were expressed directly in *E.coli* by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) which was added to a final concentration of 0.1 mM for screening in the first and second repertoires. Plasmids from clones in the third hyperimmune repertoires were transfected into HEK293 cells in the following manner: 1μl suspension of *E.coli* picked into 384 plates as single clones (CFU) were lysed by incubation for 15 minutes in 4M NaOH. Plasmid DNA was amplified using the TempliPhi Amplification kit (GE Amersham) by transferring 1μl lysed cell suspension to a mix of the kit enzyme and buffer and incubating at 30°C overnight and then

inactivating the enzyme for 10 minutes at 65°C. TempliPhi plasmids were transfected into HEK293 cells using Optimem (Invitrogen) and 293Fectin (Invitrogen) in 384 well plates in FreeStyle HEK293 medium (Invitrogen). The plates were incubated shaking at 37°C overnight and Tryptone N1 (Organotechnie) was added to all wells to a final concentration of 0.4%. Plates were incubated while shaking another 72 hours at 37°C.

Both bacterial and mammal clone supernatants were screened for activity against TT; the first repertoires were screened by ELISA and the second and third repertoire from each donor was screened by bead based fluorescence-linked immunosorbent assay (FLISA). All positive clones were identified and a number of clones were selected for sequencing (see table 1). The VH-Vκ sequences were aligned to group clones according to sequence homology. For each group, the V-D-J usage and location of somatic mutations were determined by alignment with germline sequences using the IMGT sequence directory (13).

Fab production for kinetic measurements

Fab fragments in *E.coli* were produced for SPR analysis (all clones selected for further analysis from the first repertoires and 6 and 4 clones from the second repertoire of donor TT1 and TT2, respectively) as described previously (26;27). Due to low bacterial Fab expression levels, some clone Fabs were expressed in HEK293 by cloning the Fab fragment into a mammal HEK293 expression vector, and transfecting plasmids into HEK293 cells using Optimem (Invitrogen) and 293Fectin (Invitrogen) in FreeStyle HEK293 medium (Invitrogen) in volumes of 30-100 ml. Cultures were incubated while shaking at 37°C for 6 days after which supernatants were harvested by centrifugation. Fab fragments were purified using Poly-prep columns (Bio-Rad) with protein L immobilized on agarose (Pierce) according to the manufacturer's instructions.

The concentration of purified Fab was determined by FLISA using a purified Fab as standard.

Surface plasmon resonance (SPR)

Affinities (K_D), on-rates (k_a) and off-rates (k_d) were determined as previously described (26) by SPR analysis on Biacore 2000 (GE Healthcare/Biacore, Uppsala, Sweden) or Proteon XPR36 (Bio-Rad). Briefly, TT was immobilized at low ligand densities on CM5 chips (Biacore) or GLC chips (Bio-Rad), resulting in maximum response unit (RU) values of ~100 RU or less. In most cases, this lead to proper

fitting of the data with monovalent 1:1 interaction kinetics. Fab fragments were diluted to 96 or 48 nM, and rate constants were measured by injection of at least four serial 2-fold dilutions of the antibodies at 50µl/min. Purified Fab was diluted in running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 on Biacore 2000 and PBS pH 7.2, 0.005% Tween20 on Bio-Rad) and passed over the chip at 50 µl/min.

All measurements were conducted at 25°C or 37°C. Association was measured for 5 min and dissociation for 20-30 min. However, for interactions with very slow off-rates (below $1x10^{-4}$ s^{-1}), dissociation was measured for longer periods of time, up to 16 h, depending on the off-rate. Global analysis of the serially diluted analyte responses was performed with either BIAevalution or Proteon software 2.0.

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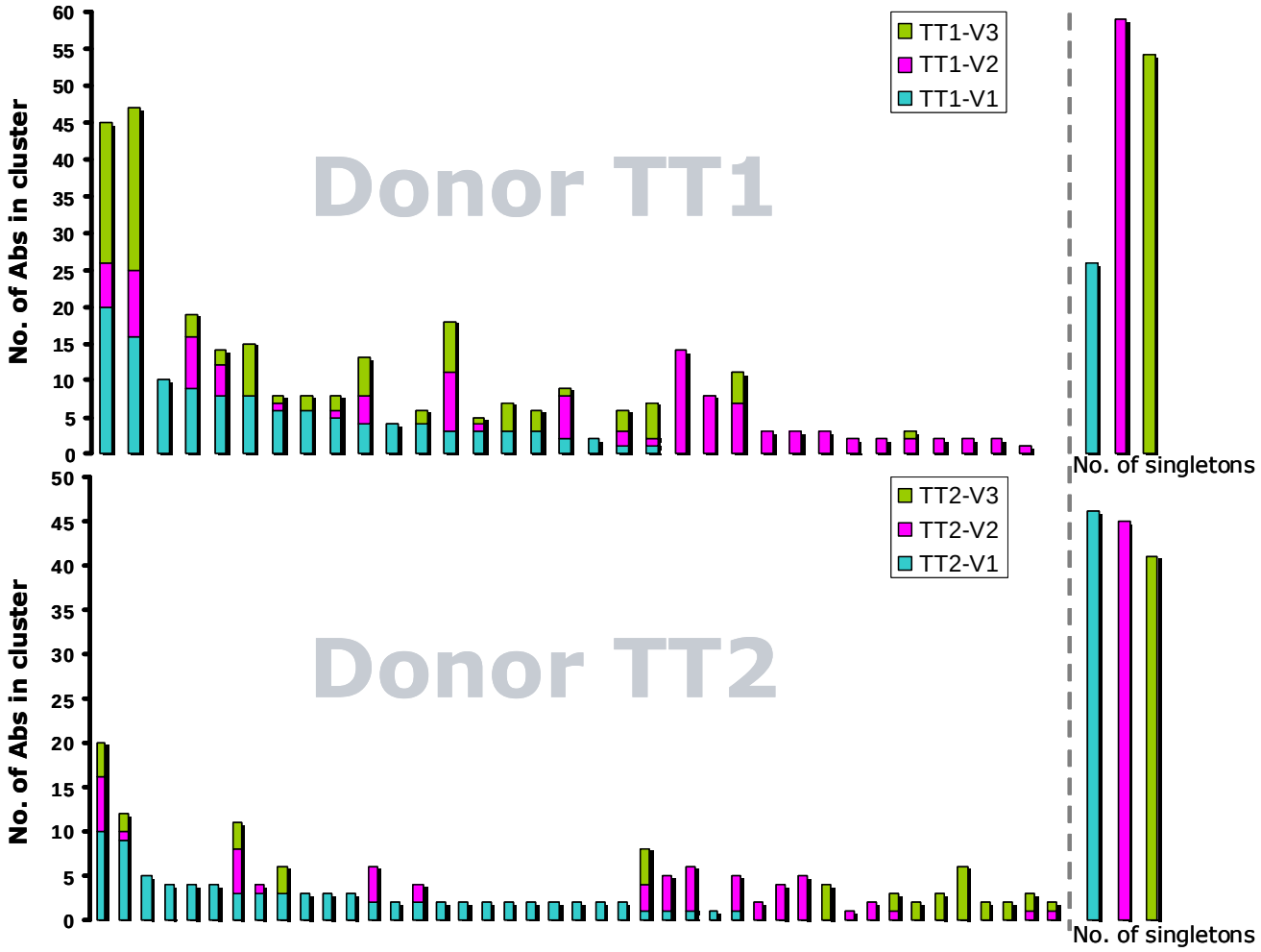
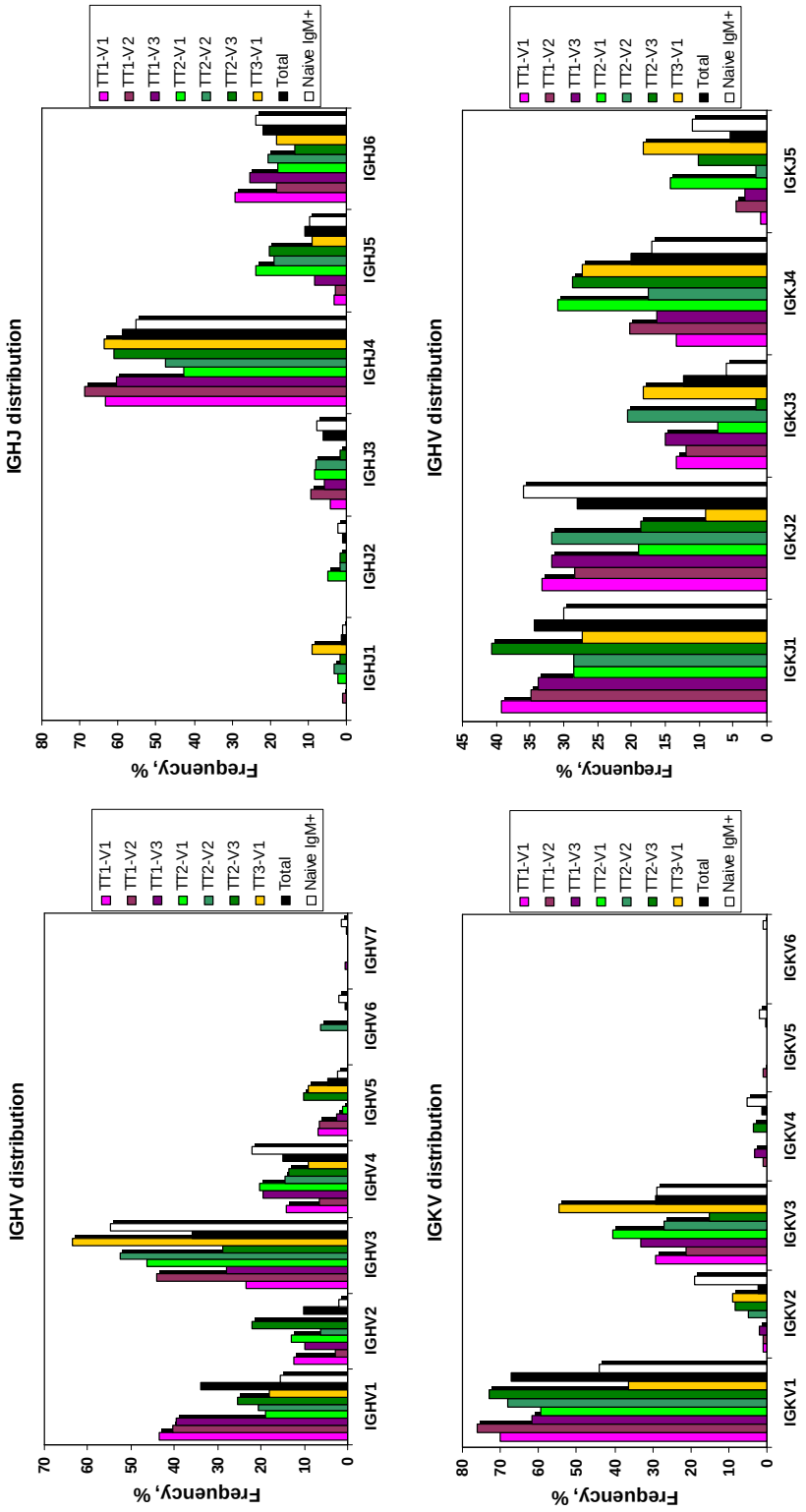
Figure 1



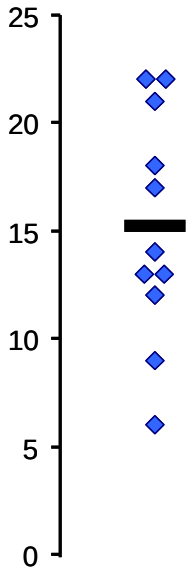
The distribution of unique antibodies in different clusters for the 3 repertoires from donor TT1 (n=382) and TT2 (n=212). Antibodies from first repertoires (V1) are shown in blue, while second (V2) and third (V3) repertoire antibodies are shown in pink and green, respectively. The number of singletons in each repertoire is shown to the right of the dashed line.

Figure 2


IGHJ distribution
IGHV distribution
IGKV distribution
IGKV distribution
Frequency, %
TT1-V1
TT1-V2
TT1-V3
TT2-V1
TT2-V2
TT2-V3
TT3-V1
Total
Naive IgM+
IGHJ1
IGHJ2
IGHJ3
IGHJ4
IGHJ5
IGHJ6
IGKJ1
IGKJ2
IGKJ3
IGKJ4
IGKJ5
IGHV1
IGHV2
IGHV3
IGHV4
IGHV5
IGHV6
IGHV7
IGKV1
IGKV2
IGKV3
IGKV4
IGKV5
IGKV6

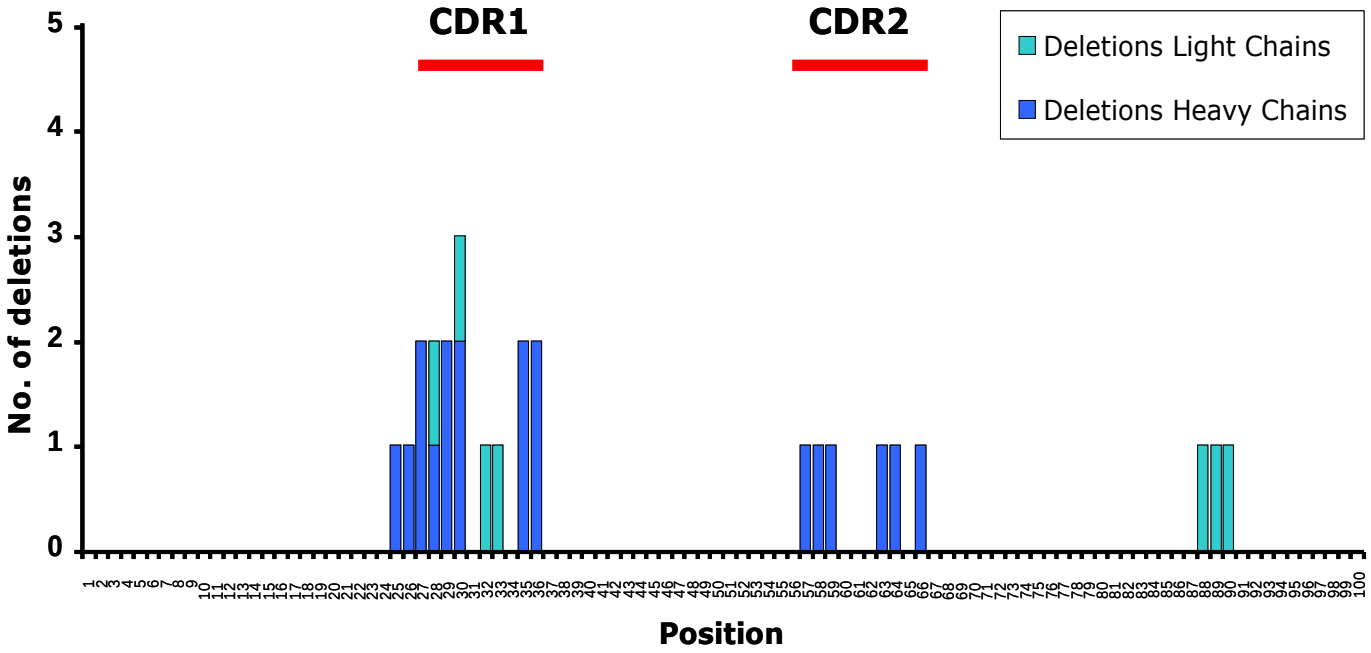
V and J gene segment usage of unique sequences. Distribution of heavy chain gene segment usage, VH (A) and JH (B), and light chain gene segment usage, VK (C) and JK (D) in unique sequences of the three consecutive antibody repertoires of TT1 (TT1-V1 n=120, TT1-V2 n=108, and TT1-V3 n=154, purple colors) and TT2 (TT2-V1 n=84, TT2-V2 n=69, and TT2-V3 n=59, green colors), and in the primary repertoire of TT3 (TT3-V1 n=11, yellow). The sum of all antibodies is indicated by black bars. White bars represent previously described naive peripheral IgM^+ B cell repertoires {Brezinschek, 1997, J. Clin. Invest.; Foster, 1997, J. Clin. Invest.} (n_{VH}=350, n_{JH}=206, $n_{VK,JK}$=321).

Figure 3



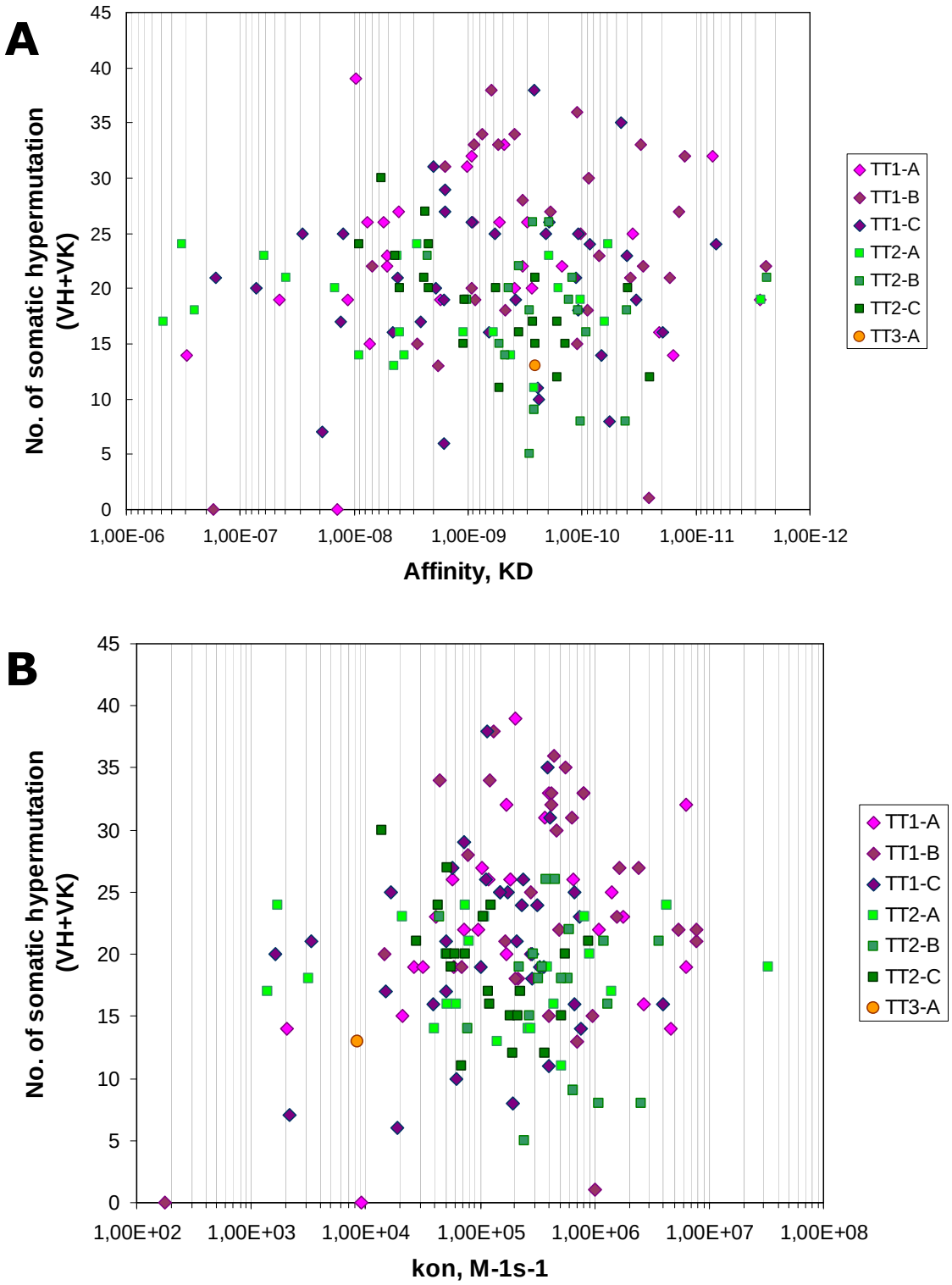
Number of hypermutations on amino acid level in heavy and light chains from 11 antibodies isolated from a virgin donor. The mean is represented by a black bar.

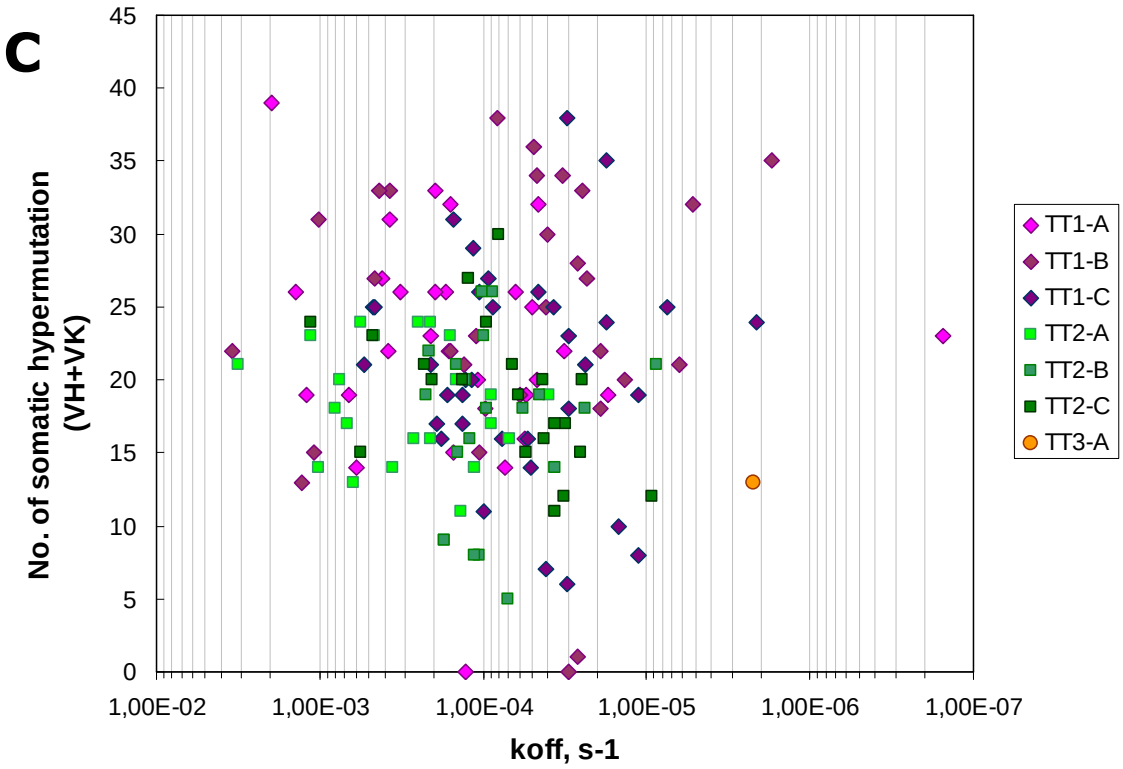
Figure 4



Deletions in the tetanus repertoires.
Positions of deleted amino acids for each clonotype are shown in dark blue for heavy chains and light blue for light chains.

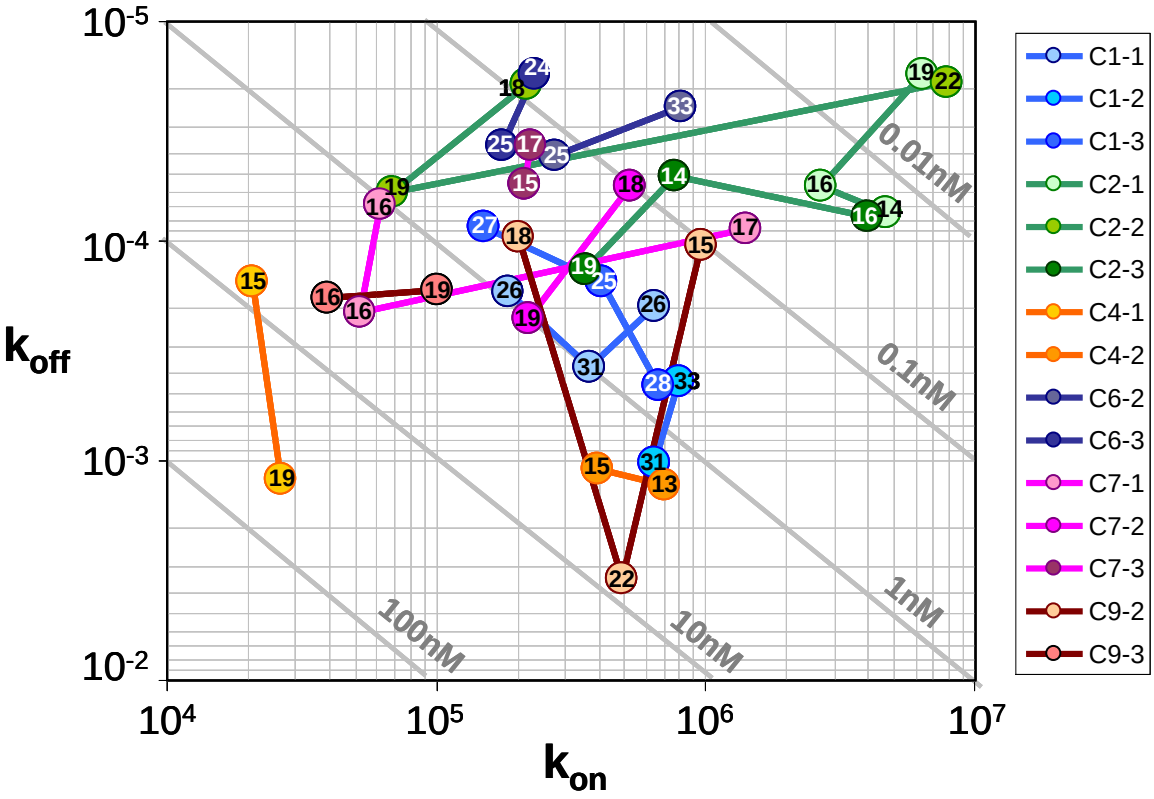
Figure 5


A
No. of somatic hypermutation (VH+VK)
45
40
35
30
25
20
15
10
5
0
1,00E-06
1,00E-07
1,00E-08
1,00E-09
1,00E-10
1,00E-11
1,00E-12
Affinity, KD
TT1-A
TT1-B
TT1-C
TT2-A
TT2-B
TT2-C
TT3-A
B
No. of somatic hypermutation (VH+VK)
45
40
35
30
25
20
15
10
5
0
1,00E+02
1,00E+03
1,00E+04
1,00E+05
1,00E+06
1,00E+07
1,00E+08
kon, M-1s-1
TT1-A
TT1-B
TT1-C
TT2-A
TT2-B
TT2-C
TT3-A



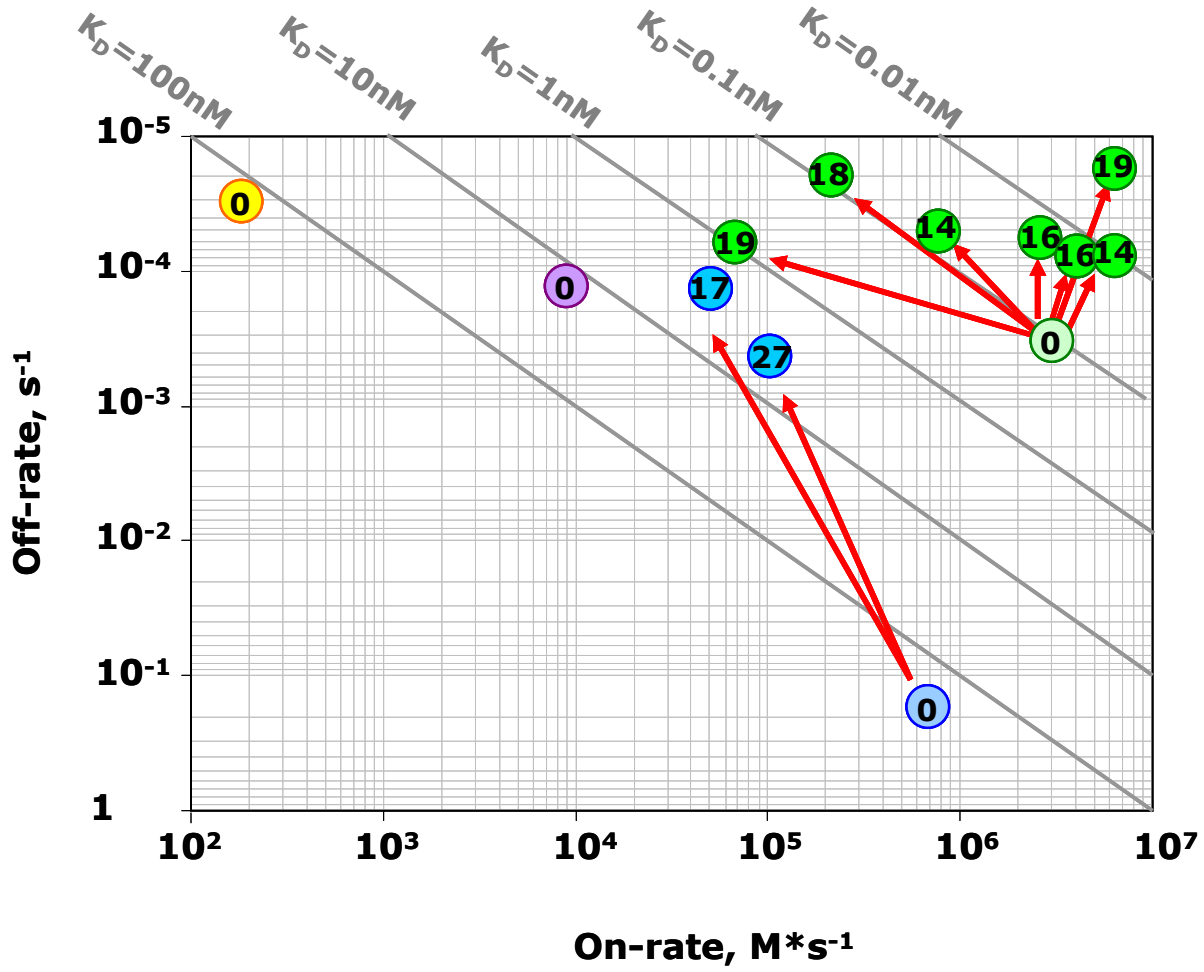
Correlation of the number of hypermutations of each antibody and its affinity (A), on-rate (B), and off-rate (C).

Figure 6



Antibody kinetics of single antibodies from 6 large clusters where members were found in at least two repertoires from a donor. The color of each circle indicates the cluster number and repertoire number (e.g. C6-2 means cluster 6, repertoire 2). The color of the connecting lines indicates the cluster (the color is shared for the different repertoires within the same cluster). The number within each circle indicates the number of amino acid hypermutations for H+L of the antibody in question. The crossing grey lines indicate the affinity (K_D) in nM. Clusters 1, 2, 4, 6 and 9 are from donor TT1 while cluster 7 is from donor TT2.

Figure 7



Kinetics of antibodies in germline configuration (non-hypermutated) or their hypermutated variants. The color of each circle indicates the cluster and the number within each circle indicates the number of amino acid hypermutations for H+L of the antibody in question. The green and the blue germline antibody are synthesized antibodies in deduced germline configuration whereas the yellow and purple germline antibodies were isolated from donor TT1 (from repertoire 1 and 2, respectively). The red arrows indicate the change in kinetics for the hypermutated variants isolated from TT1 of two of the germline antibodies. We did not isolate any hypermutated variants of the two germline antibodies isolated directly from one of the donors.